BIOACTIVITY AND CHEMICAL INVESTIGATIONS OF *PERESKIA BLEO* AND *PERESKIA GRANDIFOLIA*

SIM KAE SHIN

THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF SCIENCE

UNIVERSITY OF MALAYA

KUALA LUMPUR

2010

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: (I.C/Passport No:)

Registration/Matric No:

Name of Degree:

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

Field of Study:

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date

Subscribed and solemnly declared before,

Witness's Signature Date

Name:
Designation:

BIOACTIVITY AND CHEMICAL INVESTIGATIONS OF *PERESKIA BLEO* AND *PERESKIA GRANDIFOLIA*

ABSTRACT

Ethnopharmacological data has been one of the common useful criteria in drug discovery. *Pereskia bleo* and *Pereskia grandifolia* (Cactaceae), commonly known as 'Jarum Tujuh Bilah' in Malay and 'Cak Sing Cam' in Chinese have long been used as natural remedies in Malaysia. The experimental approach in the present study was based on bioassay-guided fractionation. The crude methanol and fractionated extracts of both *Pereskia spp.* were initially investigated for their biological activities such as antioxidant, antimicrobial and cytotoxic effect against five human cancer cell lines, namely nasopharyngeal epidermoid carcinoma cell line (KB), cervical carcinoma cell line (CasKi), colon carcinoma cell line (HCT 116), hormone-dependent breast carcinoma cell line (MCF7), lung carcinoma cell line (A549) and the non-cancer human fibroblast cell line (MRC-5) using *in vitro* cytotoxicity assay, in order to identify the bioactive extracts of both *Pereskia spp.*

The hexane and ethyl acetate extracts of both *Pereskia spp.* generally showed stronger antioxidant activities than the other extracts. Ethyl acetate extracts of both *Pereskia spp.* also showed some mild antimicrobial activities against the tested bacteria. In the cytotoxicity assay, both *Pereskia spp*. exerted no damage to MRC-5 normal cells. The ethyl acetate extracts of both *Pereskia spp.* in general gave higher inhibition and stimulation values against various cancerous cell lines compared to other extracts. The cell deaths of the selected cancer cells elicited by the cytotoxic active extracts of both *Pereskia spp.* were found to be apoptotic in nature based on a clear indication of DNA fragmentation, which is a hallmark of apoptosis. In addition, the LUX RT-qPCR [real-

time reverse transcriptase–polymerase chain reaction (RT-qPCR) using LUX (Light Upon eXtension) primers] analysis showed that apoptosis elicited by the cytotoxic extracts on selected cancer cells was mediated by p53, caspase-3 and c-myc activation in different expression levels.

Methyl palmitate, methyl linoleate, methyl α-linolenate and phytol were identified from the hexane extract of *Pereskia bleo* by GCMS analysis whilst methyl palmitate, methyl linoleate, methyl α-linolenate and methyl stearate were identified from the hexane extract of *Pereskia grandifolia*. From the results of the biological screenings, it is observed that the ethyl acetate extracts generally have stronger biological activities than other extracts. Further chemical investigations were thus directed to the ethyl acetate extracts of both *Pereskia spp.*

2,4-Di-tert-butylphenol (**1**), α-tocopherol (**2**), phytol (**3**), ß-sitosterol (**4**), dihydroactinidiolide (**5**) and a mixture of sterols containing campesterol (**6**), stigmasterol (**7**) and β-sitosterol (**4**) were isolated from *Pereskia bleo* whilst 2,4-di-tert-butylphenol (**1**), α-tocopherol (**2**), ß-sitosterol (**4**) and mixture containing methyl palmitate (**9**), methyl oleate (**10**), methyl stearate (**11**) and 2,4-di-tert-butylphenol (**1**) were isolated from *Pereskia grandifolia*. It is interesting to note that 2,4-ditert-butylphenol (**1**), α-tocopherol (**2**) and ß-sitosterol (**4**) were isolated from the ethyl acetate extracts of both *Pereskia spp.* The cytotoxic activities of the isolated constituents were evaluated against the above human cell lines and further studies on their mode of action suggested that these activities are connected with induction of apoptosis.

In addition, the toxicity of both *Pereskia spp.* was evaluated *in vivo* and were considered safe in acute oral toxicities in experimental mice. The screening of the

locally grown *Pereskia bleo* and *Pereskia grandifolia* indicated the presence of alkaloids, but the concentration was very low.

The findings of *Pereskia bleo* and *Pereskia grandifolia* in the present study provided scientific validation on the use of the leaves of both *Pereskia spp.* for the treatment of cancer. Further studies on the mutagenic and toxicity effect over a longer period of time involving detection of effects on vital organ functions should be carried out to ensure that the plants are safe for human consumption.

KAJIAN BIOAKTIVITI DAN KIMIA

PERESKIA BLEO* DAN *PERESKIA GRANDIFOLIA

ABSTRAK

Data etnofarmakologi merupakan salah satu kriteria umum yang berguna dalam penemuan sesuatu ubat tertentu. *Pereskia bleo* dan *Pereskia grandifolia* (Cactaceae) yang biasa dikenali sebagai 'Jarum Tujuh Bilah' di kalangan orang Melayu dan 'Cak Sing Cam' di kalangan orang Cina telah lama digunakan sebagai jamu semulajadi di Malaysia. Eksperimen dalam penyelidikan ini dijalankan berdasarkan pemfraksian berpanduan bioasei. Kajian awal bioaktiviti ekstrak mentah serta fraksi daripada kedua-dua spesies *Pereskia*, seperti kesan antioksidan, antimikrob dan kesitotosikan telah dikaji untuk mengenalpasti ekstrak bioaktif bagi kedua-dua spesies tersebut. Penyaringan aktiviti kesitotoksikan telah dijalankan menggunakan asei kesitotoksikan *in vitro* ke atas lima titisan sel karsinoma manusia, iaitu titisan sel karsinoma epidermoid nasofarinks (KB), titisan sel karsinoma serviks (CasKi), titisan sel karsinoma kolon (HCT 116), titisan sel karsinoma payu dara yang melibatkan hormon (MCF7), titisan sel karsinoma paru-paru (A549) serta titisan sel manusia bukan karsinoma (MRC-5).

Ekstrak heksana dan etil asetat bagi kedua-dua spesies *Pereskia* secara umumnya menunjukkan aktiviti antioksidan yang lebih kuat daripada ekstrak lain. Ekstrak etil asetat bagi kedua-dua spesies *Pereskia* juga menunjukkan aktiviti antimikrob yang lemah pada bakteria yang dikaji. Bagi asei kesitotoksikan, ekstrak kedua-dua spesies *Pereskia* tidak aktif ke atas sel normal MRC-5. Ekstrak etil asetat kedua-dua spesies *Pereskia* secara umumnya menunjukkan nilai rintangan dan stimulasi yang lebih tinggi ke atas pelbagai titisan sel kanser berbanding dengan ekstrak

lain. Kematian kanser sel yang dirangsang oleh ekstrak aktif kedua-dua spesies *Pereskia* telah didapati mengaruh kematian sel secara apoptosis berdasarkan tanda fragmentasi DNA yang jelas. Fragmentasi DNA merupakan ciri utama apoptosis. Tambahan lagi, analisis LUX RT-qPCR [*real-time reverse transcriptase–polymerase chain reaction (RT-qPCR) using LUX (Light Upon eXtension) primers*] menunjukkan apoptosis yang dirangsang oleh ekstrak aktif ke atas sel kanser tertentu adalah menerusi pengaktifan p53, caspase-3 dan c-myc pada tahap ekspresi yang berbeza.

Metil palmitat, metil linoleat, metil α-linolenat dan fitol telah dikenalpasti daripada ekstrak heksana *Pereskia bleo* melalui analisis GCMS manakala metil palmitat, metil linoleat, metil α-linolenat dan metil stearat telah dikenalpasti daripada ekstrak heksana *Pereskia grandifolia*. Daripada hasil penyaringan biologi, ekstrak etil asetat secara umumnya didapati mempunyai aktiviti biologi yang lebih kuat daripada ekstrak yang lain. Kajian kimia seterusnya ditumpukan kepada ekstrak etil asetat bagi kedua-dua spesies *Pereskia*.

2,4-Di-tert-butilfenol (**1**), α-tokoferol (**2**), fitol (**3**), ß-sitosterol (**4**), dihidroaktinidiolid (**5**) dan satu campuran sterol yang mengandungi kampesterol (**6**), stigmasterol (**7**) and β-sitosterol (**4**) telah dipisahkan dan dikenalpasti daripada *Pereskia bleo* sementara 2,4-di-tert-butilfenol (**1**), α-tokoferol (**2**), ß-sitosterol (**4**) dan satu campuran sebatian yang mengandungi metil palmitat (**9**), metil oleate (**10**), metil stearat (**11**) dan 2,4-di-tert-butilfenol (**1**) telah dipisahkan dan dikenalpasti daripada *Pereskia grandifolia*. Perlu diambil perhatian bahawa 2,4-di-tert-butilfenol (**1**), α-tokoferol (**2**) dan ß-sitosterol (**4**) wujud dalam ekstrak etil asetat kedua-dua spesies *Pereskia*. Kajian aktiviti kesitotosikan sebatian ke atas titisan sel kanser dan penyelidikan selanjutnya ke atas mod tindakan, mencadangkan aktiviti sitotosik sebatian adalah berkaitan dengan induksi apoptosis.

Di samping itu, ketoksikan kedua-dua spesies *Pereskia* telah dikaji secara *in vivo* dan hasil eksperimen ketoksikan akut yang dijalankan ke atas tikus menunjukkan ekstrak tumbuhan adalah selamat. Penyaringan untuk *Pereskia bleo* dan *Pereskia grandifolia* tempatan menunjukkan kehadiran sejumlah kecil alkaloid sahaja.

Hasil kajian terhadap *Pereskia bleo* dan *Pereskia grandifolia* ini memberi fakta saintifik yang sah dalam penggunaan daun kedua-dua *Pereskia* spesies untuk pengubatan kanser. Kajian mutagenik dan kesan ketoksikan pada organ penting untuk jangka yang lebih panjang perlu dijalankan untuk memastikan tumbuhan tersebut adalah selamat bagi penggunaan manusia.

.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation and gratitude to my supervisors, Datin Professor Dr. Sri Nurestri Abdul Malek from Institute of Biological Sciences, Faculty of Science and Datin Professor Dr. Norhanom Abdul Wahab from Centre for Foundation Studies in Science, for their continuous supervisions, advices, guidance and continuous encouragements throughout my project. I thank them especially for their patience and the time spent in discussing various approaches and results.

I would also like to express my acknowledgment and appreciation to Dato' Professor Dr. Hashim Yaacob from International University College of Nursing for the selection of the plant species and his invaluable advices in publishing the findings.

My utmost appreciation to Associate Professor Dr. Kim Kah Hui from Department of Physiology, Faculty of Medicine for making available the facilities in his laboratory for the animal study. Special thanks to Mr V.T. Johgalingam for his willingness in teaching me basic toxicity research techniques.

My appreciation also goes to Associate Professor Koshy Philip from Institute of Biological Sciences, Faculty of Science for the use of laboratory facilities to carry out the antimicrobial screenings. I would also like to thank Mr Saravana Kumar for his guidance in antimicrobial screening work.

I would like to gratefully acknowledge Madam Hong Sok Lai and Mr Lee Guan Serm for their guidance and advice in chemical investigations. My acknowledgement is

also extended to Miss Syarifah Nur Syed Abdul Rahman and Miss Gowri Kanagasabapathy for their help, patience and motivation in carrying out the laboratory experiments.

My sincere appreciation to Miss Sujata Ramasamy for her assistance and generous encouragements in cell culture work. My gratitude towards Miss Wong Kah Hui, Miss Fathiah Abdullah, Miss Maizatul Azmah, Mr Mohd Azrul Mohd Sari, Miss Chan Pek Yue, Miss Lai Li Kuan, Miss Law Ing Kin and Miss Veronica Alicia Yap for their moral support and friendship in providing a good working environment.

My heartfelt gratitude to Mr Yong Yean Kong, Miss Tan Hong Yien, Miss Ong Lai Yee, Miss Teh Siew Phooi, Miss Lee Yeat Mei, Mr Dino Tan Bee Aik and Dr. Andrew Lim for all the help, guidance and patience in the gene expression work, and especially access to the facilities in the laboratory. Special thanks to Mr Yong Yean Kong for sharing all the knowledge in molecular genetics. He has been particularly helpful with suggestions on molecular studies for this work and has never been short of useful comments, ideas and solutions when approached with difficulties.

In addition, I am also very grateful to the staff of the Faculty of Science, Mr Ghana, Puan Latipah, Mr Asokan, Mr Teo, Pak Din, Encik Rufli and Encik Khamis for their support in carrying out the project.

Special thanks to Miss Chaw Sook Ting, Miss Pauline Ong, Miss Stephanie Lau Chiau Hwee and Miss Lim Siew Hua for invaluable moral support, encouragement and many useful comments on my work.

Last but not least, my deepest appreciation and love to my husband, Dr Lai Kim Leng for his understanding and endless patience, as well as my parents, sisters and my family-in-law for their encouragements and support which has inspired me to accomplish this study.

I would like to acknowledge the Ministry of Higher Education (MOHE) and University of Malaya for the Skim Latihan Akademik IPTA (SLAI) award that kept me financially sound throughout the study period. This research project was supported by research funds from University of Malaya (PJP F0155/2005D, PPP PS056/2007C, PS241/2008C) and MOHE (FRGS FP040/2008C).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

ANOVA	ANalysis Of VAriance
ATCC	American Tissue Culture Collection
A549	Human lung carcinoma cell line
BHA	Butylated hydroxyanisole
BLAST	Basic Local Alignment and Search Tool
CasKi	Human cervical carcinoma cell line
CC	Column chromatography
$CDCl_3$	Deuterated chloroform
cDNA	Complementary deoxyribonucleic acid
CH_2Cl_2	Dichloromethane
$CHCl_3$	Chloroform
CO_2	Carbon dioxide
Ct	Threshold cycle
℃	Degree Celsius
DEPC	Diethyl pyrocarbonate
DEPT	Distortionless Enhancement by Polarisation Transfer
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic triphosphate
DPPH	1,1-Diphenyl-2-picrylhydrazyl
ds	Double stranded
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
EtOAc	Ethyl acetate
FAM	6-carboxy-fluorescein
FBS	Foetal Bovine Serum
g	Gram
GCMS	Gas Chromatography Mass Spectroscopy
HCT 116	Human colon carcinoma cell line
HEPES	N-2-Hydroxylethyl-Piperazine-N-2-Ethane-Sulfonoc
HPLC	High Pressured Liquid Chromatography
h	Hour

H_2O	Water
IC_{50}	Inhibition Concentration at 50 %
IR	Infra Red
JOE	6-carboxy-4',5'-dichloro-2',7'-dimethoxy-flurescein
KB	Human nasopharyngeal epidermoid carcinoma cell line
kg	Kilogram
L	Litre
LD_{50}	Lethal Dose at 50 %
LUX	Light Upon eXtension
µg	Microgram
µl	Microlitre
µm	Micrometer
MBC	Minimum Bacterial Concentration
MCF7	Hormone-dependent breast carcinoma cell line
MeOH	Methanol
Mg	Milligram
$MgCl_2$	Magnesium chloride
MIC	Minimum Inhibitory Concentration
min	Minute
ml	Millilitre
Mm	Millimetre
MRC-5	Non-cancer human fibroblast cell line
mRNA	Messenger RNA
MS	Mass Spectroscopy
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
$NaHCO_3$	Sodium bicarbonate
NMR	Nuclear Magnetic Resonance
nm	Nanometre
NRTC	Non-reverse-transcriptase control
NTC	Non-template control
OD	Optical Density
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction

Prep-TLC	Preparative-Thin Layer Chromatography
qPCR	Real-time polymerase chain reaction
QSTD	Quantitative standard
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotation per minute
RT-PCR	Reverse-transcriptase polymerase chain reaction
RT-qPCR	Real-time reverse-transcriptase polymerase chain reaction
s	Second
SD	Standard deviation
spp.	Species
ss	Single stranded
T_A	Annealing temperature
TBE buffer	Tris-Borate-EDTA buffer
T_m	Melting temperature
Taq	*Thermus aquaticu*
TLC	Thin Layer Chromatography
TUNEL	Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling
UV	Ultraviolet
%	Percentage

CHAPTER 1

INTRODUCTION

Today, naturally derived products play an important role as source of medicine. Many pharmaceutical agents have been discovered by screening natural products from plants and marine organisms. The structural diversity of compounds derived from natural products provides valuable sources of novel lead compounds against newly discovered therapeutic targets (Harvey, 1999).

The statistics regarding the uses of natural products as drug are now well-known and have been repeatedly presented and discussed. Over the period 1981-2002, 1031 new chemical entities (NCEs) have been discovered. Thus, in the area of cancer the percentage of new chemical entities that are non-synthetic has remained at 62 % averaged over the whole time frame. In the antihypertensive area, of the 74 formally known synthetic drugs, 48 can be traced to natural product structures/ mimics (Newman *et al.*, 2003; Abas, 2005).

There are around 250,000 plant species in the world and 60 % of them are located in the tropical rainforests. The plant resources of Malaysia comprise about 15,000 species of higher plants. It was estimated that about 1,000 of these plants have undergone simple chemical screening and much less have been subjected to thorough chemical or pharmacological studies (Goh *et al.*, 1993). The huge diversity of the Malaysian flora means that we can expect well diversed chemical structures from their secondary metabolites, and chemical diversity is one of the plus factors that makes natural products excellent candidates for any screening programme.

Ethnopharmacological data has been one of the common useful ways for the discovery of biological active compounds from plants, in which the selection of a plant is based on the prior information on the folk medicine use of the plant. It is generally known that ethnomedical data provides substantially increased chance of finding active plants relative to random approach (Chapuis *et al*., 1988; Cordell *et al*., 1991).

Pereskia bleo (*P. bleo*) and *Pereskia grandifolia* (*P. grandifolia*), commonly known as the 'Jarum Tujuh Bilah' in Malay and 'Cak Sing Cam' in Chinese belong to the botanical family Cactaceae. *P. bleo* can be easily mistaken from *P. grandifolia* because they look similar vegetatively. However, they can be distinguished by the leaves, flowers and spines. *P. bleo* have thinner and corrugated leaves, orangish-red flowers with shorter spines compared to *P. grandifolia*. In contrast, *P. grandifolia* have thicker and uncorrugated leaves, pink to purple-pink flowers with longer and lesser spines.

Both *P. bleo* and *P. grandifolia* have been used as natural remedy in cancer-related diseases, either eaten raw or taken as a concoction brewed from fresh plant. The leaves are also taken as vegetables by some natives. Both are believed to have anti-cancer, anti-tumour, anti-rheumatic, anti-ulcer and anti-inflammatory properties. They are also used as remedy for the relief of headache, gastric pain, ulcers, haemorrhoids and atopic dermatitis; and refresh the body (Goh, 2000; Rahmat, 2004; Tan *et al.*, 2005). The pounded leaf paste of *P. bleo* is also applied to the wound or cut for pain relief (Kehidupan Sihat, 2006). In Panama, the locals use the whole plant of *P. bleo* to treat the gastrointestinal problems (Gupta *et al*., 1996). On the other hand, *P. grandifolia* is also used to reduce swellings (Sahu *et al.*, 1974; Anon, 1969).

Although *P. bleo* and *P. grandifolia* are reported to be used in a large number of Malaysian traditional medicine preparations, there is not much recorded data on biological studies and chemical investigations of *P. bleo* and *P. grandifolia*. There is only one phytochemical report (Doetsch *et al.*, 1980) and four biological studies (Matsuse *et al.*, 1999; Tan *et al.*, 2005; Ruegg *et al.*, 2006; Er *et al*., 2007) reported for *P. bleo*. Similarly, very little phytochemical work (Doetsch *et al.*, 1980; Sahu *et al.*, 1974) and biological study (Ooi *et al.*, 2003) has been reported for *P. grandifolia*.

The experimental approach in the present study is based on bioassay-guided fractionation. In this endeavour, the crude methanol and fractionated extracts of *P. bleo* and *P. grandifolia* were firstly prepared for the biological assessment. The extracts were subjected to antioxidant assays, antimicrobial assays and neutral red cytotoxicity assay to identify the bioactive extracts of both *Pereskia* species. The cytotoxic active extracts were further subjected to detection of DNA fragmentation (apoptosis) and determination of the expression level of apoptotic–related genes to verify the possible mechanisms of cell death elicited by the extracts on the cells.

After identifying the bioactive extracts of *P. bleo* and *P. grandifolia*, the chemical constituents responsible for the bioactivities were identified. Thus, the bioactive extracts were subjected to isolation and purification procedures to obtain chemical constituents present in the extracts. The isolated chemical constituents were further tested for their cytotoxic activity against the selected human cell lines. The active cytotoxic chemical constituents were then subjected to detection of apoptosis and determination of the expression level of apoptotic–related genes.

Acute oral toxicity was also undertaken in the present study to determine the safety parameters of the leaves of both *Pereskia spp.* as *in vitro* trials did not always

reflect the outcome of *in vivo* studies. In addition, the locally grown *P. bleo* and *P. grandifolia* were screened for alkaloids in the present study to confirm the presence of alkaloids as Doetsch *et al.* (1980) reported the isolation of alkaloids in both *Pereskia spp*. The general procedures in the present study are outlined in Figure 1.1.

In the present study, the antioxidant activities of the extracts were determined by four different assays, namely scavenging activity of plant extracts on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals, reducing power assay, ß-carotene method and Folin-Ciocalteau's method. To our knowledge, there is no antioxidant study reported for both *P. bleo* and *P. grandifolia*.

The antimicrobial activities of the extracts were determined by agar diffusion and broth dilution method. The broth dilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). To our knowledge, there is only one report on the antimicrobial properties of *P. bleo* (Ruegg *et al.*, 2006) and no such report on *P. grandifolia*.

Determination of cytotoxic effect of *Pereskia spp.* extracts and isolated chemical constituents were investigated on six human cell lines, which were the human nasopharyngeal epidermoid carcinoma cell line (KB), human cervical carcinoma cell line (CasKi), human colon carcinoma cell line (HCT 116), hormone-dependent breast carcinoma cell line (MCF7), human lung carcinoma cell line (A549) and non-cancer human fibroblast cell line (MRC-5). To our knowledge, no report on the cytotoxicity of *P. bleo* and *P. grandifolia* against the above mentioned human cancer cell lines has ever been published.

Apoptosis or programmed cell death plays important roles in many biological processes including carcinogenesis, tumorigenesis and cancer. Many chemotherapeutic

and chemopreventive agents have been found to induce apoptotic cell death (Kong *et al*., 2001). In the present study, DNA fragmentation (apoptosis) was detected using a modified TUNEL [terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling] assay.

Subsequently, the protocol for the evaluation of mRNA expression levels of apoptosis-related genes by LUX RT-qPCR [real-time reverse transcriptase–polymerase chain reaction (RT-qPCR) using LUX (Light Upon eXtension) primers] analysis has been developed in the present study. This LUX RT-qPCR assay offers significant advantages with respect to the rapidity, sensitivity and reproducibility of quantification assay for gene expression.

Objectives of study

The main objectives of the present study were as follows:

a. to screen for antioxidant, antimicrobial and *in vitro* cytotoxic activities of *P. bleo* and *P. grandifolia* extracts.

b. to investigate the ability of cytotoxic active extracts of *P. bleo* and *P. grandifolia* to induce apoptosis and to determine the possible mechanisms of cell death elicited by the extracts on selected cancer cells.

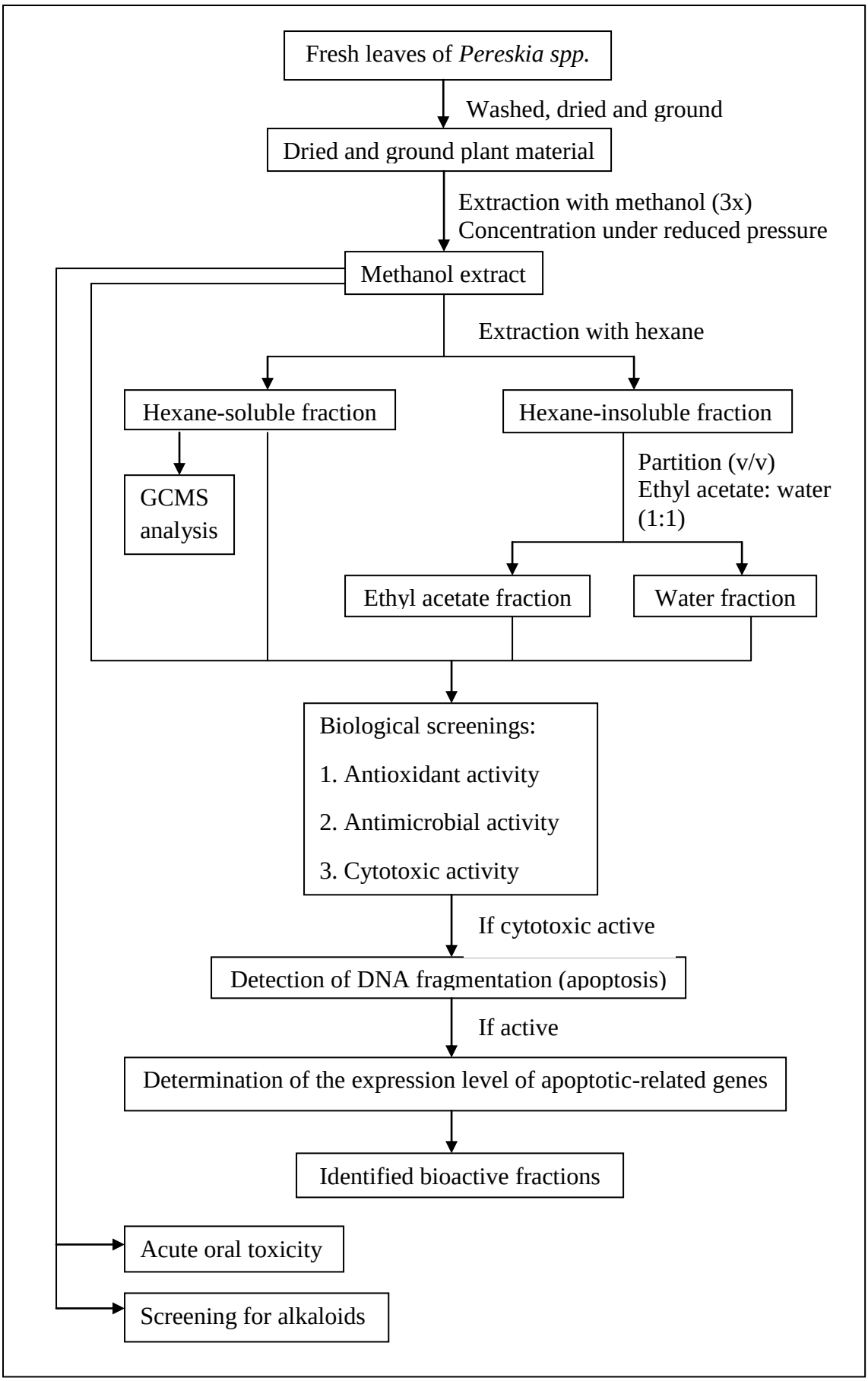
c. to isolate the chemical constituents from the identified bioactive extracts of *P. bleo* and *P. grandifolia* through the bioassay guided fractionation technique.

d. to identify and elucidate the structures of the chemical constituents by using modern spectroscopic methods.

e. to evaluate the cytotoxic activities of the chemical constituents, their ability to induce apoptosis and possible mechanisms of cell death elicited by the chemical constituents on selected cancer cells.

f. to develop a protocol for the evaluation of mRNA expression levels of apoptosis-related genes by LUX RT-qPCR assay [real-time reverse transcriptase–polymerase chain reaction (RT-qPCR) using LUX (Light Upon eXtension) primers].

g. to determine the safety parameters of *P. bleo* and *P. grandifolia* extracts by using acute oral toxicity assessment.


Fresh leaves of *Pereskia spp.*
Washed, dried and ground
Dried and ground plant material
Extraction with methanol (3x)
Concentration under reduced pressure
Methanol extract
Extraction with hexane
Hexane-soluble fraction
Hexane-insoluble fraction
GCMS analysis
Partition (v/v)
Ethyl acetate: water (1:1)
Ethyl acetate fraction
Water fraction
Biological screenings:
1. Antioxidant activity
2. Antimicrobial activity
3. Cytotoxic activity
If cytotoxic active
Detection of DNA fragmentation (apoptosis)
If active
Determination of the expression level of apoptotic-related genes
Identified bioactive fractions
Acute oral toxicity
Screening for alkaloids

……continued

Figure 1.1, continued

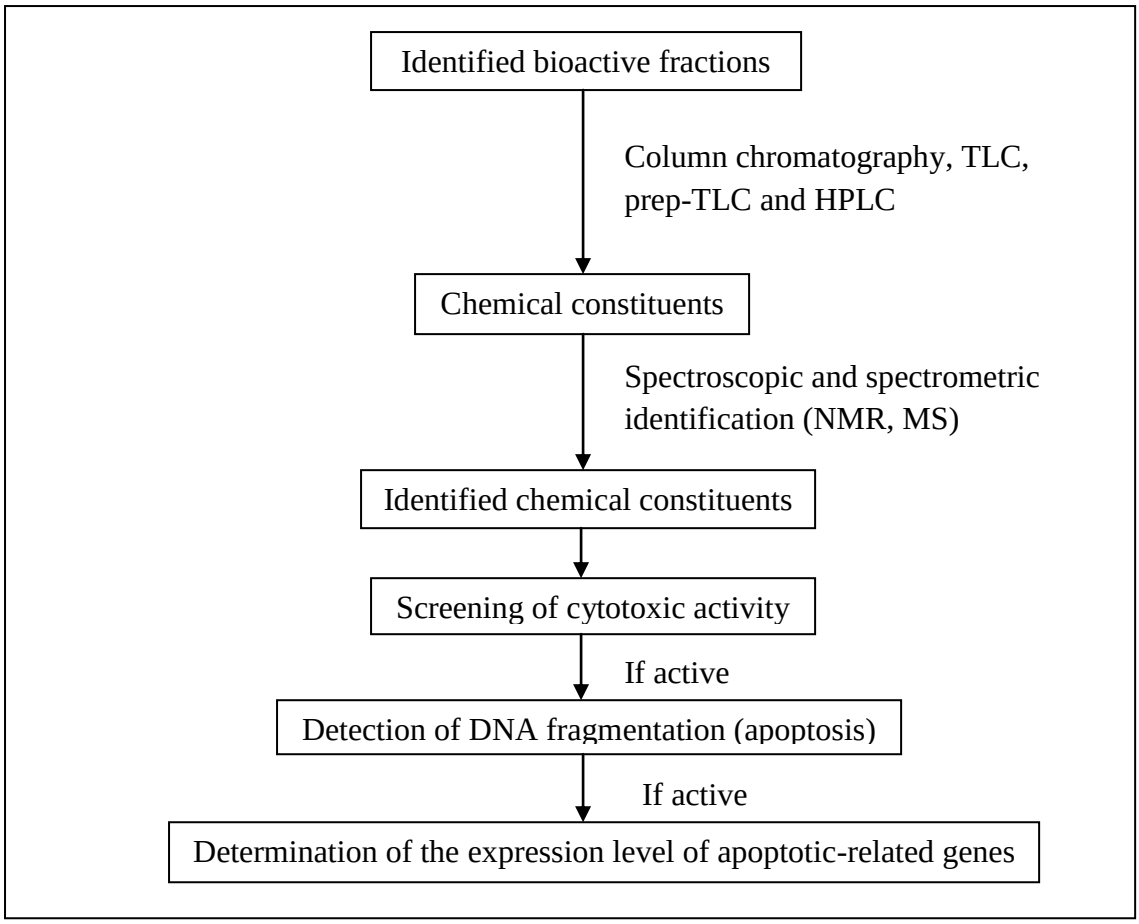


Figure 1.1: Outline of general procedures

CHAPTER 2

LITERATURE REVIEW

2.1 Natural products

Natural products usually refer to secondary metabolites which has relatively complex structures. Secondary metabolites are usually characteristic of specific botanical sources in comparison to primary metabolites which occur in almost every plant. A general characteristic of natural products is that few of them have a clearly recognized function in the metabolic activities of the organisms in which they are found (Geissman *et al.*, 1969).

Natural products can be classified into various families, such as alkaloids, terpenoids and phenolics including flavonoids. Natural products perform various functions, and many of them have interesting and useful biological activities (Harvey, 1999). The utility of natural products as a source of novel structures is still alive and well. The number of plants used as medicinal agents in commerce globally is unknown, but there are at least 1,000 species alone in China (Duke and Ayensu, 1985; Abas, 2005).

2.2 Cancer

Cancer is the general term for a series of neoplastic diseases that are characterized by changes in a cell leading to abnormal (unordered and uncontrolled) cellular proliferation (Pettit, 1997). The disorder occurs in the normal processes of cell division, which are controlled by the genetic material (DNA) of the cell. Cancers may be caused in one of three ways, namely incorrect diet, genetic predisposition, and *via* the environment (Reddy *et al*., 2003).

To date, mortality that results from the common forms of cancer is still unacceptably high. The Second Report of the National Cancer Registry of Malaysia suggested a total of 23,746 cancer cases were diagnosed among Malaysians in the year 2003, comprising 10,473 males and 13,273 females. The crude rate for males was 97.4 per 100,000 population and 127.6 per 100,000 populations for females. The age standardized incidence rate (ASR) for all cancers was 134.3 per 100,000 males and 154.2 per 100,000 females (The Second Report of National Cancer Registry, 2003).

2.2.1 Carcinogens

The majority of human cancers result from exposure to environmental carcinogens; these include both natural and manmade chemicals, radiation and viruses. Carcinogens may be divided into several classes, such as (i) genotoxic carcinogens, if they react with nucleic acids. These can be directly acting or primary carcinogens, if they are of such reactivity so as to directly affect cellular constituents; (ii) alternatively, they may be procarcinogens that require metabolic activation to induce carcinogenesis; (iii) epigenetic carcinogens are those that are not genotoxic (Reddy *et al*., 2003; Timbrell, 2000). It is also clear that genetic predisposition is one of the factors of human cancers apart from exposure to carcinogens. Thus, patients with the genetic xeroderma pigmentosum are more susceptible to skin cancer (Reddy *et al*., 2003).

Carcinogens in the diet that trigger the initial stage include moulds and aflatoxins (for example, in peanuts and maize), nitrosamines (in smoked meats and other cured products), rancid fats and cooking oils, alcohol, and additives and preservatives. A combination of foods may have a cumulative effect, and when incorrect diet is added to a polluted environment, smoking, UV radiation, free radicals,

lack of exercise, and stress, the stage is set for DNA damage and cancer progression (Reddy *et al*., 2003).

2.2.2 Cell cycle

In cells that are dividing, the nuclear DNA molecules must be duplicated and then distributed in a way that ensures the two new cells receive a complete set of genetic instructions. The cells pass through a series of discrete stages called G1 phase, S phase, G2 phase and M phase in order to accomplish these tasks (Kleinsmith, 2006). These four phases are collectively referred to as the cell cycle.

The cell cycle is commonly represented by a circular diagram (Figure 2.1). The G1, S and G2 phases are collectively referred as interphase. Besides providing the time needed for a cell to make copies of its DNA molecules, interphase is also a period of cell growth. Interphase occupies about 95 % of a typical cell cycle; whereas the actual process of cell division (M phase) only takes about 5 % (Kleinsmith, 2006). G1 is defined as the interval between M phase and S phase, and G2 is defined as the interval between S phase and M phase. S phase is defined as the time during the cell cycle when DNA synthesis is taking place, leading to a doubling of the amount of DNA per cell. M phase is the time when the amount of DNA per cell drops in half as cells divide. The restriction point is a control point near the end of G1 where the cell cycle can be halted until conditions are suitable for progression into S phase. Under normal conditions, the ability to pass through the restriction point is governed mainly by the presence of growth factors.

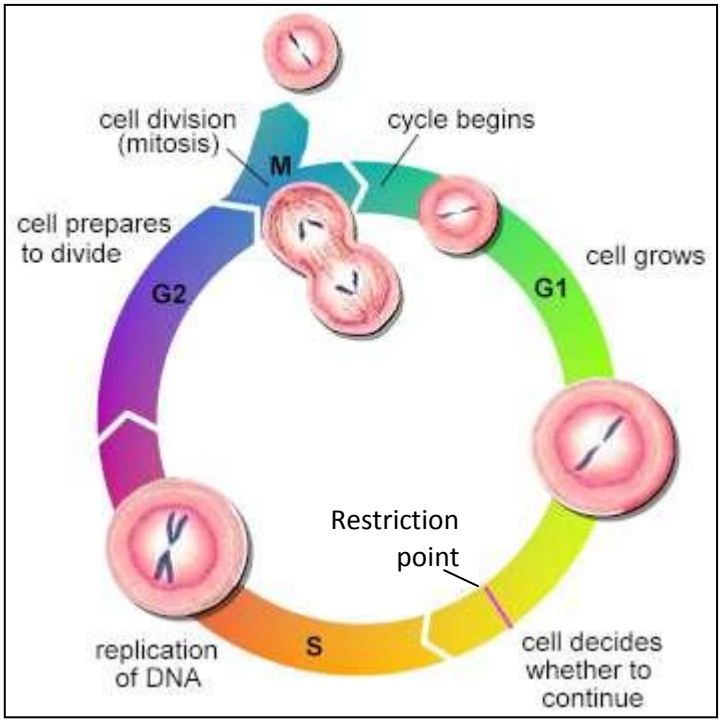


Figure 2.1: The cell cycle
(Adapted from http://herb4cancer.files.wordpress.com/2007/11/cell-cycle2.jpg, 19 August 2009)

2.2.3 Carcinogenesis

The transformation of a normal cell into a cancerous cell is believed to proceed through many stages over a number of years or even decades. Carcinogenesis is a multistep process that involves initiation, promotion and tumour progression. Initiation involves a reaction between the cancer-producing substance (carcinogen) and the DNA of tissue cells. There may be a genetic susceptibility. This stage may remain dormant, and the subject may only be at risk for developing cancer at a later stage. Promotion involves a prolonged period of proliferation of the initiated cells, occurs very slowly over a period ranging from several months to years. During this stage, a change in diet and lifestyle can have a beneficial effect so that the person may not develop cancer during his or her lifetime. The third and final stage involves progression and spread of the cancer, at which point diet may have less of an impact. Preventing initiation is an important anticancer strategy, as are the opportunities to inhibit cancer throughout the latter stages of malignancy (Reddy *et al*., 2003; Kleinsmith, 2006).

2.3 Apoptosis in cancer

Most of the cells from higher eukaryotes have the ability to self-destruct by activation of an intrinsic cellular suicide program referred to as programmed cell death or apoptosis (Ellis *et al*., 1991; Steller, 1995). Apoptosis is an intrinsic biological event that plays an essential role in development, homeostasis and in many disease process. Culling extra cells in a precise and systematic way is an important aspect of normal development. Hence, the other term for this process is programmed cell death. Occasionally, cell death program goes awry in several diseases. Degenerative diseases may result in (or be the result of) excessive apoptosis and some cancers appear to inhibit cell death cascades resulting in excessive and uncontrolled proliferation (O'Brien *et al*., 1998).

Apoptosis is a unique type of cell death, different from what happens when cells are destroyed by physical injury or exposure to certain poisons. In response to such non-specific damage, cells undergo necrosis, a slow type of death in which cells swell and eventually burst, spewing their contents into surrounding tissues. Necrosis often results in a local inflammatory reaction that can cause further cell destruction, which makes it potentially dangerous (Kleinsmith, 2006; O'Brien *et al*., 1998).

In contrast, apoptosis kills cells quickly and neatly, without causing damage to surrounding tissue. Apoptosis is characterized by certain morphological features, including reduction in cell volume, cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation (Kerr *et al*., 1972, 1994; Wyllie *et al.*, 1980; O'Brien *et al*., 1998). The process involves a carefully orchestrated sequence of intracellular events that systematically dismantle the cell (Figure 2.2).

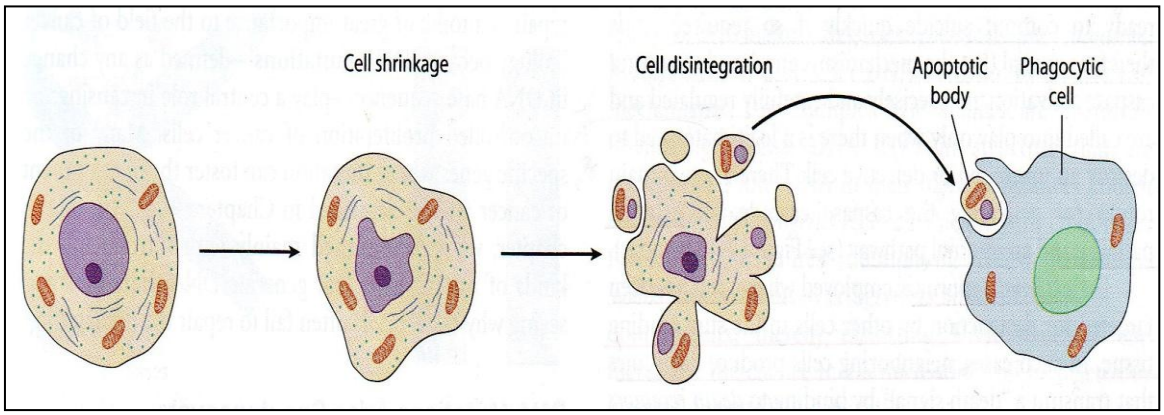


Figure 2.2: Main steps in apoptosis (Kleinsmith, 2006)

As a cell begins to undergo apoptosis, the first observable change is cell shrinkage. Next, small bubble-like protrusions of cytoplasm form at the cell membrane as the nucleus and other cellular structures begin to disintegrate. The chromosomal DNA is then degraded into small pieces and the entire cell breaks apart, forming small fragments known as membrane-bound apoptotic bodies that are engulfed by macrophages or neighbouring phagocytic cells without generating an inflammatory response. Phagocytic cells are specialized for ingesting foreign matter and breaking it down into molecules that can be recycled for other purposes (Kleinsmith, 2006).

The genetic basis for apoptosis implies that cell death, like any other metabolic or development program, can be disrupted by mutation. Elucidation of the core machinery of apoptosis has provided new insights into cancer biology, revealing novel strategies for cancer therapy (Reed, 2002). In the last decade, basic cancer research has produced remarkable advances in the understanding of cancer biology and cancer genetics. Among the most important of these advances is the realization that apoptosis and the genes that control it have a profound effect on the malignant phenotype. For example, it is now clear that some oncogenic mutations disrupt apoptosis, leading to tumour initiation, progression or metastasis. Conversely, compelling evidence indicates

that other oncogenic changes promote apoptosis, thereby producing selective pressure to override apoptosis during multistage carcinogenesis (Scott and Athena, 2000).

Review the process of apoptosis

Apoptosis involves a dynamic interplay of several molecules with up-regulatory and down-regulatory properties. Stimulation of pro-apoptotic molecules or inhibition of anti-apoptotic factors is dependent on the cell type and the form of insult. It is unlikely that the activation or inactivation of a single component will alter the ultimate fate of the cell and lead to apoptosis (Huerta *et al.*, 2007).

In the classical form of apoptosis, apoptosis is carried out by a series of protein-degrading enzymes known as cysteinyl aspartate-specific proteases (caspases). Caspases are grouped into initiator caspases (8 and 10) and execution caspases (3, 6 and 7) (Kleinsmith, 2006; Huerta *et al.*, 2007).

Normally, caspase reside in cells in the form of inactive precursors called procaspases. When a cell receives a signal to commit suicide, an initiating member of the procaspase family is converted into an active caspase. The activated caspase catalyzes the conversion of another procaspase into an active caspase, which activates yet another procaspase and so forth (Kleinsmith, 2006). Hence, we can conclude that apoptosis is carried out by a caspase cascade.

The presence of procaspase within a cell means that the cell is programmed with the seeds of its own destruction, ready to commit suicide quickly if required. It is therefore crucial that the mechanisms employed to control caspase activation are precisely and carefully regulated and are called into play only when there is a need to destroy unneeded or defective cells (Kleinsmith, 2006). Activation of caspase cascade occurs *via* two main routes, which are external pathway and/or internal pathway. Figure 2.3 shows the two main routes for triggering apoptosis.

Generally, the external pathway is employed when a cell has been targeted for destruction by other cells in the surrounding tissue. In such cases, neighbouring cells produce molecules that transmit a 'death signal' by binding to death receptors present on the outer surface of the targeted cell. The activated death receptors then interact with, and trigger activation of, initiator procaspase molecules located inside the cell, thereby starting the caspase cascade (Figure 2.3) (Kleinsmith, 2006; Huerta *et al.*, 2007).

The internal pathway is a pathway particularly relevant to the field of cancer biology, which functions mainly in the destruction of cells that have sustained extensive DNA damage. In the internal pathway, damaged DNA triggers accumulation of the p53 protein, which stimulates the production of death-promoting proteins that alter the permeability of mitochondrial membranes. This event leads to the release of a group of mitochondrial proteins, including cytochrome c that activates the caspase cascade and thereby cause the cell to be destroyed by apoptosis.

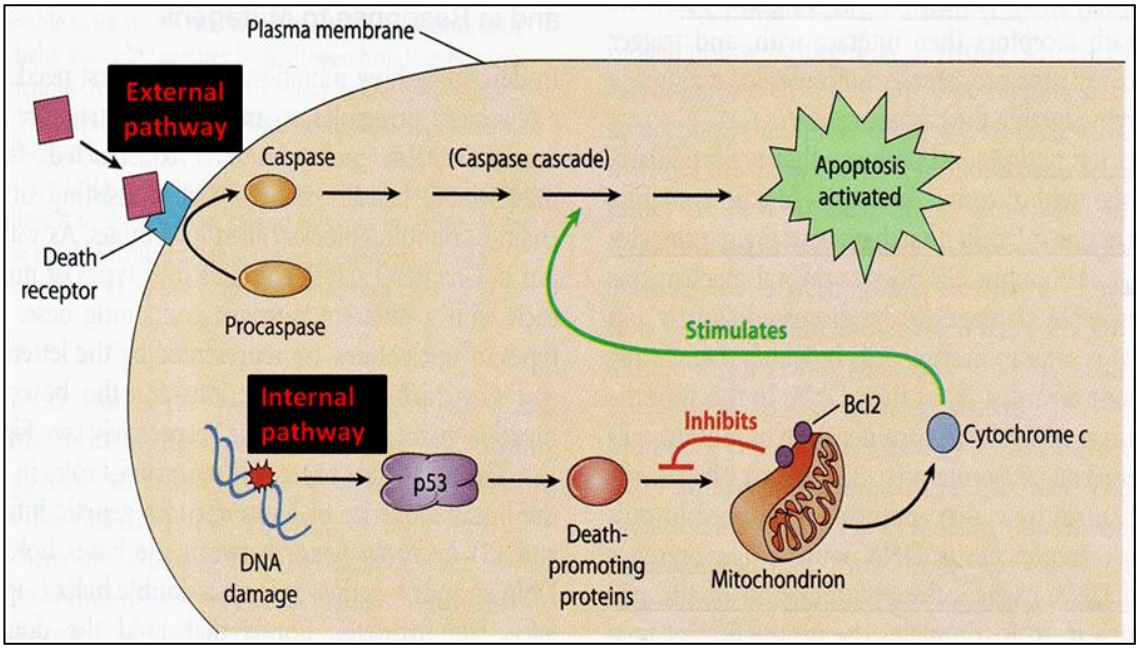


Figure 2.3: The two main routes for triggering apoptosis (Adapted from Kleinsmith, 2006)

(i) External pathway

The external pathway is also known as extrinsic pathway or death receptor pathway. The detail mechanism of external pathway is shown in Figure 2.4. In Figure 2.4, it is clearly shown that the external pathway is initiated by binding of: 1) the TNF-ligand (tumour necrosis factor ligand) to the TNF receptor, 2) the TRAIL-ligand (TNF-related apoptosis inducing ligand) to the DR (death receptors), and 3) the FAS-ligand (apoptosis inducing receptor ligand) to the FAS receptors. This association leads to recruitment of adaptor molecules such as FADD [FAS (apoptosis inducing receptor) Associated Death Domain] or TRADD (Tumour Associated Death Domain) resulting in activation of initiator caspases (caspase 8 and 10), which in turn cleaves and activates executioner caspases (caspase 3, 6 and 7), culminating in apoptosis (Walczak and Krammer, 2000; Huerta *et al.*, 2007).

(ii) Internal pathway

The internal pathway is also known as intrinsic pathway or mitochondrial pathway. The internal pathway (Figure 2.4) is activated by release of cytochrome c from the mitochondrial inter-membrane into the cytosol. Once released, cytochrome c interacts with Apaf-1 (apoptosis activating factor-1), ATP and procaspase 9 to form apoptosome. The apoptosome cleaves and activates caspase 9, which leads to caspase 3, 6 and 7 activity stimulating apoptosis (Adrain and Martin, 2001).

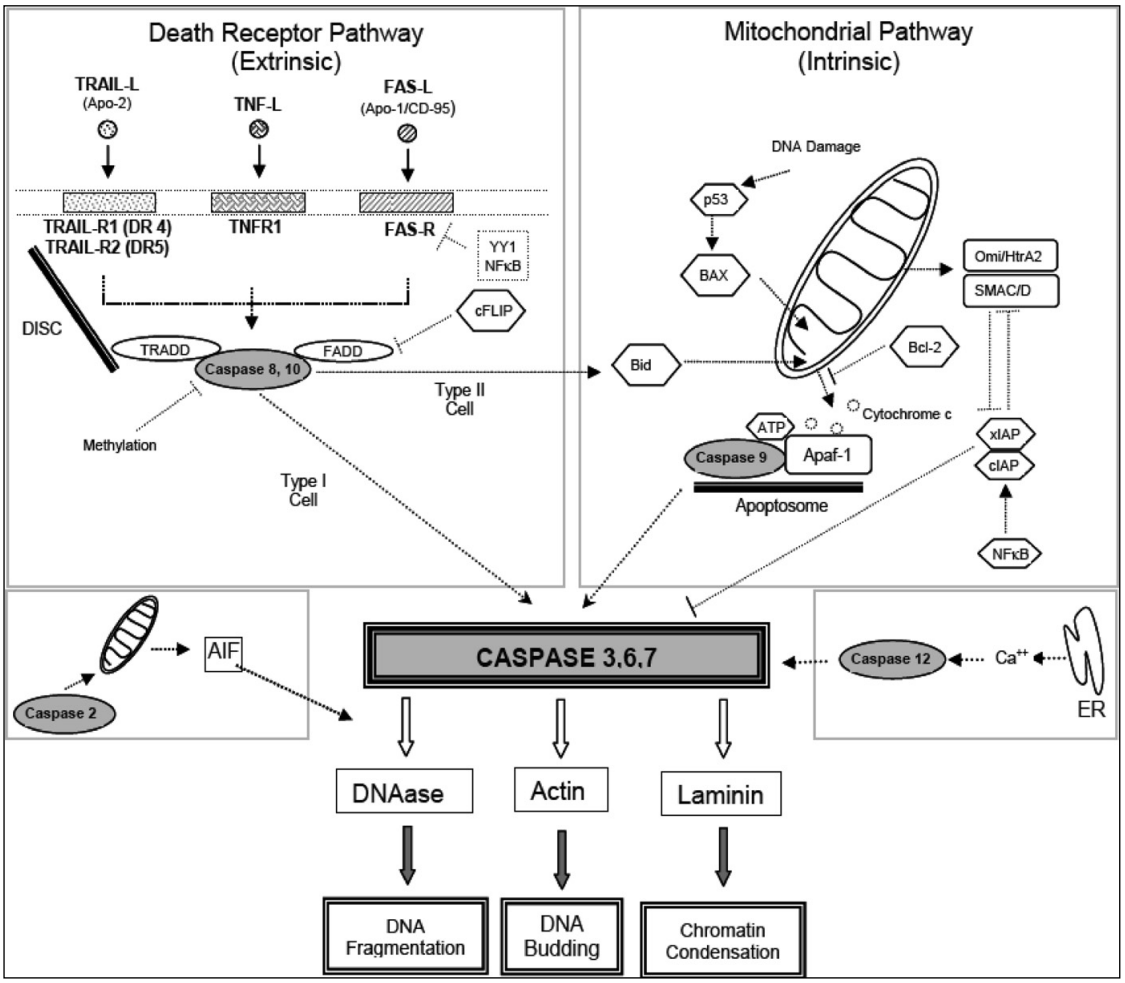


Figure 2.4: Mechanism of apoptosis (Huerta *et al.*, 2007)

In summary, apoptosis occurs in a sequential manner with the first morphological changes appearing at the membrane. Cell to cell adhesion decreases and either the cytosolic or mitochondrial proteins are altered resulting in nuclear changes. The ultimate determinant of apoptosis is an orderly form of intranucleosomal DNA fragmentation. As the biochemical hallmark of apoptosis is initiation of caspase cascade that leads to DNA fragmentation, both caspase activation and DNA fragmentation need to be demonstrated to establish if a cell has undergone the process of apoptosis (Huerta *et al.*, 2007).

2.4 Natural products and defence against carcinogenesis

Researchers are increasingly turning their attention to folk medicine looking for new leads to develop better drugs against cancer, as well as viral and microbial infections (Galal *et al*., 1991; Hoffmann *et al*., 1993). Natural products have provided an endless supply of active compounds as source of medicine. A major group of these products are the powerful antioxidants, which are usually phenolic in nature, whilst some include reactive groups that confer protective properties (Reddy *et al*., 2003). Many pharmaceutical agents have been discovered by screening natural products from plants, animals, marine organisms and microorganisms.

Plants are excellent sources of macronutrients (carbohydrates, proteins, fats and fibres) and micronutrients (antioxidants, vitamins and trace minerals). In addition, they are sources of an amazing diversity of secondary metabolites (phytochemicals), which are not essential for normal bodily function but are still biologically active and of medicinal value (Deorukhkar *et al.*, 2007). Although the mechanism of the protective effects of the natural products might not be clear, the fact that the consumption of fruits and vegetables lowers the incidence of carcinogenesis at a wide variety of sites is broadly supported. The epidemiological evidence suggests fruits and vegetables protection against a wide array of cancers, particularly those of the respiratory and digestive tracts to a lesser extent the hormone-related cancers (Reddy *et al*., 2003).

The chemical diversity, structural complexity, affordability, lack of substantial toxic effects and inherent biologic activity of natural products makes them ideal candidates for new therapeutics. Natural products not only disrupt aberrant signalling pathways leading to cancer (i.e. proliferation, deregulation of apoptosis, angiogenesis, invasion and metastasis) but also synergize with chemotherapy and radiotherapy (Deorukhkar *et al.*, 2007). About 25 % of the drugs prescribed worldwide come from plants, 121 such active compounds being in current use. Of the 252 drugs considered as

basic and essential by the World Health Organization (WHO). 11 % are exclusively of plant origin and a significant number are synthetic drugs obtained from natural precursors (Rates, 2001). Vincristine, irinotecan, etoposide and paclitaxel are examples of plant-derived compounds that are being employed in cancer treatment (Rocha *et al*., 2001). It is estimated that 60 % of anti-tumour and infectious drugs already on the market or under clinical trial are of natural origin (Shu, 1998).

2.5 Natural products with conventional therapeutic modalities

Cancer therapy often involves a multi-pronged approach that incorporates surgical, chemotherapeutic and radiotherapeutic strategies. It is increasingly evident that the success of these interventions is hampered by development of resistance to these therapies and the inability to continue these therapies indefinitely due to their cumulative toxicities. One of the strategies that merit consideration in this battle against cancer is that of combining these traditional therapies with non-toxic agents that augment their effectiveness by selectively sensitizing cancer cells to the cytotoxic effects of chemotherapy and /or radiotherapy (Garg *et al*., 2005).

According to Deorukhkar *et al.* (2007), natural products exert their anticancer effects at different levels, thereby modulating both the process of tumourigenesis and the efficacy of anticancer therapies. At level one, natural products inhibit the initial processes of tumourigenesis by inhibiting different steps that govern the transformation of an initiated cancer cell to a premalignant lesion. These are chiefly dietary phytochemicals and the role they play is chemopreventive. At level two, natural products exert their anticancer effects on multiple processes that result in the progression of premalignant lesions into overtly malignant tumours. At level three, natural products can be combined with conventional therapeutic modalities in order to achieve better tumour cell kill and to overcome the resistance of tumours to these

therapies. For example, (i) natural products enhance the apoptotic cell death and/or clonogenic death induced by chemotherapy and/or radiotherapy; (ii) natural products abrogate various pro-survival signalling pathways upregulated by tumours in an effort to overcome the cell triggered by chemotherapy and radiotherapy; (iii) natural products could minimize the toxicity of conventional therapies by inhibiting the physiologic processes induced by these therapies within normal tissues. The inherent lack of toxicity of natural products and the potential reduction of toxicity of cytotoxic therapies could improve the therapeutic ratio of cancer treatment.

2.6 The Cactaceae family

The cacti are well-known desert plants, widely recognized by their specialized growth form and essentially leafless condition. The Cactaceae is the only family of the order and contains from 50 to 150 genera and about 2000 species renowned for their remarkable morphological and physiological adaptations to drought (Barthlott & Hunt, 1993). The plants are xerophytes and, possibly one exception, are all native to the Americas. They will not grow where there is virtually no rainfall, but thrive in deserts where there is a reasonable rainfall even if rain occurs very infrequently. Some cacti occur in rain forests, where they are often epiphytes (Evans, 1996).

The leaves of the Cactaceae have evolved into spines, in order to allow less water to evaporate through transpiration than regular leaves, defend the cactus against water-seeking animals. Photosynthesis is carried out by enlarged stems, which also store water. Unlike many other succulents, the stem is the only part of a true cactus where this takes place. Very few members of the family have leaves, and when present these are usually rudimentary and soon fall off; they are typically awl-shaped and only 1-3 mm long. Two genera, *Pereskia* and *Pereskiopsis*, do however retain large, non-succulent leaves 5-25 cm long and also non-succulent stems. *Pereskia*, a genus that

consists of 17 species of leafy shrubs and trees, has now been determined to be the ancestral genus from which all other cacti evolved.
(http://en.wikipedia.org/wiki/Cactaceae, 20 July 2008)

Cacti come in a wide range of shapes and sizes. Some grow to great size. Some cacti produce beautiful flowers, which like spines and branches arise from areoles. The flowers are mostly bisexual and actinomorphic and commonly have many weakly differentiated perianth segments arising from an epigynous zone. The androecium typically consists of a very large number of stamens arising from the inner face of the epigynous zone. The gynoecium consists of a compound pistil of 3-many carpels, an equal number of stigmas, and an equal number of parietal placentae with numerous ovules in the single locule of the inferior ovary. Many cactus species are night-blooming, as they are pollinated by nocturnal insects or small animals, principally moths and bats. Cacti range from small and round to pole-like and tall, such as the Saguaro. Some cacti bear edible fruit. The fruit is a berry, often with spines or bristles. A number of cactus species are cultivated for use as houseplants, as well as for ornamental gardens nowadays.
(http://www.biologie.uni-hamburg.de/b-online/vascular/cact.htm, 06 July 2006 and http://en.wikipedia.org/wiki/Cactaceae, 20 July 2008).

2.7 The *Pereskia* genus

The genus *Pereskia* comprises of 25 tropical species and varieties of leafy cacti. They originate from the regions extending from Brazil in the South to Mexico in the North. The genus is named after Nicolas Fabre de Peiresc, a French botanist of the 16th century and has been given its own subfamily Pereskioideae. Members of this genus are usually referred to as lemon vines or rose cacti or leaf cacti, though this latter also refers to the genus *Epiphyllum*.

Species of *Pereskia* are very unusual cacti, resembling more wild roses than actual cacti. *Pereskia* species have large, bright green, privet-like leaves and long spiny stems. They are not always succulent plants; they can be classified as shrubs, climbing plants or slightly succulent trees. Unlike *Pereskiopsis*, *Maihuenia*, *Quiabentia* and *Austrocylindropuntia* (which possess succulent, persistent leaves), *Pereskia* is the only cactus genus that has persistent non-succulent leaves.

The species of *Pereskia* are easy to grow. They grow fast and flower prolifically. Shrub species usually reach 1 m high, but climbing or arborescent species can even reach 5 to 20 m. Flowers may appear alone or in clusters. They generally resemble roses and reach a diameter of 1 to 5 cm. Colours of the flower depend on each species and vary from white, yellow to magenta or red. Fruits are ordinarily spherical, of 2 to 5 cm diameter, and are wine red when ripe (http://en.wikipedia.org/wiki/Pereskia, 16 April 2006).

Scientific classification

Kingdom : Plantae

Division : Magnoliophyta

Class : Magnoliopsida

Order : Caryophyllales

Family : Cactaceae

Subfamily : Pereskioideae

Genus : *Pereskia* Mill

2.7.1 The *Pereskia bleo*

Pereskia bleo (Kunth) DC., commonly known as the 'Jarum Tujuh Bilah' in Malay and 'Cak Sing Cam' in Chinese belongs to the botanical family Cactaceae. The appearance of *P. bleo* is shown in Figure 2.5-2.8.

P. bleo has several synonyms, such as *Pereskia corrugata*, *Pereskia cruenta*, *Pereskia panamensis*, *Rhodocactus corrugatus*, *Rhodocactus bleo*, cactus bleo, wax rose cactus, orange rose cactus and Pereskia rose cactus (http://www.desert-tropicals.com/Plants/Cactaceae/Pereskia_bleo.html, 07 July 2006). Although the plants resemble wild roses, they are actually primitive cacti with leaves. The stems are not succulent but woody, and have prominent spines.

P. bleo can reach up to 12 feet (3 m) in height and a width of about half its height, but can also be kept pruned into a bushy shrub. The fleshy dark green leaves can reach 8 inches (20 cm) long by 2.5 inches (6.5 cm) wide. The 2 inch (5 cm) orangish-red flowers open late in the afternoon and last only one day. They are very showy. If fertilized, they create a waxy hemispherical yellow fruit that is quite conspicuous. *P. bleo* likes full sun and dry conditions and propagation is by mean of cuttings. (http://toptropicals.com/cgi-bin/garden_catalog/cat.cgi?uid=pereskia_bleo, 16 April 2006 and http://www.plantoftheweek.org/week219.shtml, 16 April 2006).

P. bleo has been used as a natural remedy, either eaten raw (two fresh leaves per day) or taken as a concoction brewed from fresh plant (seven fresh flowers or leaves). The leaves were also taken as vegetable by some natives. *P. bleo* is believed to have anti-cancer, anti-tumour, anti-rheumatic, anti-ulcer and anti-inflammatory activities. It is also used as remedy for the relief of gastric pain and refreshment of the body (Goh, 2000; Tan *et al.*, 2005). In Panama, the locals use the whole plant of *P. bleo* to treat gastrointestinal problems (Gupta *et al*., 1996).

In Malaysia, the Chinese use *P. bleo* for the treatment of cancer and cancer-related diseases, like breast carcinoma, brain and uterine tumours. A decoction of the leaves is drunk for relief of headache, gastric pain, ulcers, haemorrhoids and atopic dermatitis. The pounded leaf paste may be applied to the wound or cut for pain relief (Kehidupan Sihat, 2006).

Figure 2.5: The appearance of *P. bleo* in Seksyen 17, Petaling Jaya, Selangor, Malaysia

Figure 2.6: The stem of *P. bleo*

Figure 2.7: The hemispherical fruit of *P. bleo*

Figure 2.8: The orangish-red flowers of *P. bleo*

Previous work on *P. bleo*

Although the *P. bleo* is reported to be used in a large number of Malaysian traditional medicine preparations, there is not much recorded data on biological studies or cytotoxic activity against various cancer cell lines. Chemical investigations on *P. bleo* are also rare in comparison to other *Pereskia* species, as there were only one phytochemical report and four biological studies reported for this plant.

The earliest phytochemical study was by Doetsch *et al.* (1980), who reported the isolation of four alkaloids, namely 3,4-dimethoxy-β-phenethylamine, mescaline, 3-methoxytyramine and tyramine (Figure 2.12).

A later report by Matsuse *et al.* (1999) tested the water and methanol extracts of *P. bleo* for anti-human immunodeficiency virus (HIV) effects by determining the inhibition of HIV-1 induced cytopathic effect (CPE) on MT-4 cells. However, the *P. bleo* extracts did not show any positive anti-HIV effects in the screening.

An investigation by Tan *et al.* (2005) reported that the methanol extract of *P. bleo* possessed cytotoxic effects against T-47D (human breast carcinoma cell line) cells and the cell death was found to be apoptotic in nature, mainly *via* the activation of the caspase-3 and c-myc pathways. Ruegg *et al.* (2006) reported that the methanol and dichloromethane extracts of the aerial part of *P. bleo* did not show active antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Mycobacterium smegmatis*, *Candida albicans*, *Pseudomonas aeruginosa* and *Helicobacter pylori*.

A more recent investigation by Er *et al*. (2007) indicated the anti-proliferative and mutagenic activities of aqueous and methanol extracts of *P. bleo* leaves against 4T1 (mouse mammary cancer cell line) cells or NIH/3T3 (normal mouse fibroblast cell line) cells. The authors also reported that there was an increase in the level of apoptosis in 4T1 and NIH/3T3 cells treated with increasing concentrations of the aqueous extract of *P. bleo*. This suggested that cell death occurred *via* apoptosis to some degree in the aqueous extract-treated cells.

2.7.2 The *Pereskia grandifolia*

Pereskia grandifolia Haw. is a shrub or small tree that can grow up to 10 m tall. The trunk is very spiny and measures up to 20 cm in diameter. Unlike most cacti, *Pereskia* species have persistent leaves and also have spines (modified leaves); however, they lack glochids. *P. grandifolia* has one leaf below each areole, and (uniquely) some leaves are among the spines within the areole. The leaves are ovate or obovate-lanceolate; usually 9-23 cm long and 4-6 cm broad, rather thin, venation pinnate. The spines are 0-8, 1-4 cm long, brown-black, straight, slender. The appearance of *P. grandifolia* is shown in Figure 2.9-2.11.

In contrast to *P. bleo*, *P. grandifolia* has beautiful pink to purple-pink flowers. The flowers are few to many, diurnal, 3-7 cm in diameter; the floral cup and fruit combine into a fleshy structure with an inferior ovary. The fruit is pear-shaped, irregularly angled, to 10 cm long, and to 7 cm in diameter, fleshy. Oddly, the fruit have floral areoles which are bracteate and short woolly hairs; usually the bracts fall before the fruit is mature. *P. grandifolia* is cultivated widely in tropical countries and is native to Brazil (http://www.doacs.state.fl.us/pi/enpp/94-7&8all.htm, 07 July 2006; Benson, 1982; Huxley, 1992).

In Malay, *P. grandifolia* is known as 'Jarum Tujuh Bilah', 'Duri Bintang Tujuh', 'Jarum Bilah' or 'Pokok 1001 Khasiat'. In Chinese, it is known as 'Cak Sing Cam' (Chen, 2002). *P. grandifolia* has several synonyms or other Latin names, such as *Rhodocactus grandifolius* (Haw.) Kunth., *Rhodocactus tampicanus* (Weber.) Backeb., *Pereskia ochnacarpa* Miq. and *Pereskia tampicana* F.A.C.Weber. It is also known as large flowered Barbados gooseberry, large leaved Barbados gooseberry, rose cactus, grootblaar and Barbadosstekelbessie (http://www.hear.org/gcw/html/autogend/species/14349.HTM, 07 July 2006).

P. grandifolia can either be eaten raw (two fresh leaves per day) or taken as a concoction brewed from fresh plant (seven fresh flowers or leaves). *P. grandifolia* is believed to have anti-cancer, anti-tumor, anti-rheumatic, anti-ulcer and anti-inflammatory activities. It is also used as remedy for the relief of gastric pain and refreshes the body (Goh, 2000). *P. grandifolia* is also used to reduce swellings (Sahu *et al.*, 1974 and Anon, 1969).

Rahmat (2004) reported that *P. grandifolia* is used by the locals to give relief to headaches, besides its use in preventing cancer and refreshing the body. Chen (2002) reported that the Chinese boil the leaves alone or together with meat to be eaten as soup. It can fight any type of cancer from head to toe and also help in curing high blood pressure, diabetes, gastric problems, headaches and pain relief. In Brazil, the translation of *P. grandifolia* is 'Pray-For-Us', and the leaves can either be used in green salads, or be cooked (http://davesgarden.com/pf/go/57249/index.html, 26 June 2006).

Figure 2.9: The appearance of *P. grandifolia* in Seksyen 17, Petaling Jaya, Selangor

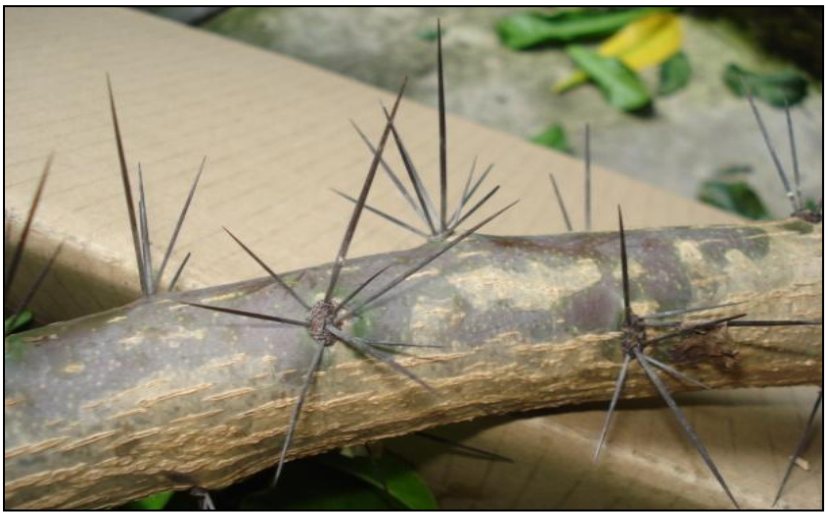


Figure 2.10: The stem of *P. grandifolia*

Figure 2.11: The pink flowers of *P. grandifolia*

Previous work on *P. grandifolia*

As for *P. bleo*, very little phytochemical work and biological study has been reported for *P. grandifolia*. Doetsch *et al.* (1980) isolated three alkaloids, *p*-methoxy-β-hydroxy-β-phenethylamine, 3-methoxytyramine and tyramine from *P. grandifolia* (Figure 2.12). Investigation by Sahu *et al.* (1974) yielded the dimethyl ester (1a) from dried powdered fruits of *P. grandifolia* (Figure 2.13). The only biological report on *P. grandifolia* by Ooi *et al.* (2003) stated that the leaves extract was found to inhibit the

cell growth of NCI-II23 (human non-small lung adenocarcinoma cell line) and Caov-3 (human ovarian carcinoma cell line) cells.

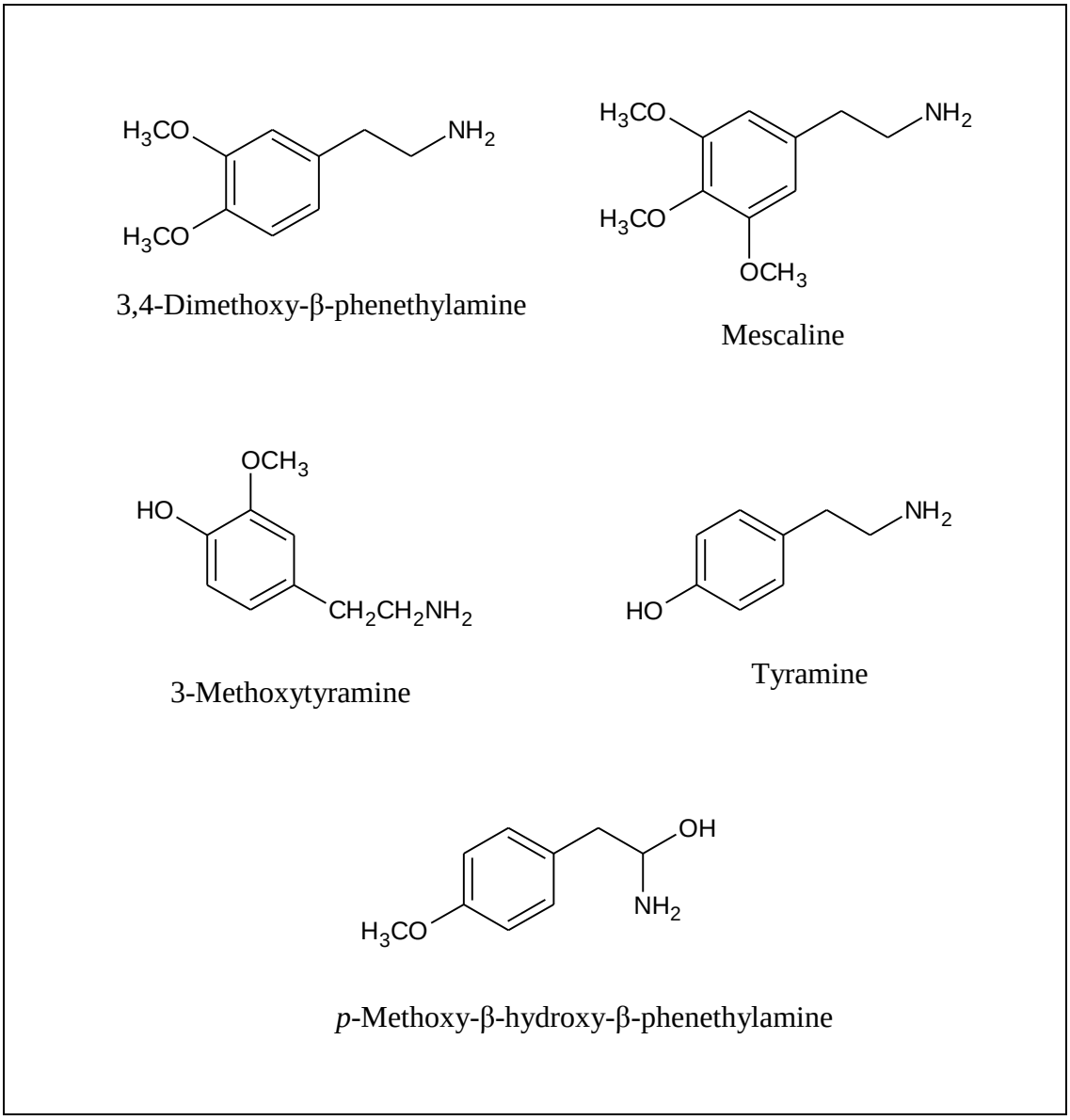


Figure 2.12: Alkaloids isolated from *P. bleo* and *P. grandifolia* (Doetsch *et al.*, 1980)

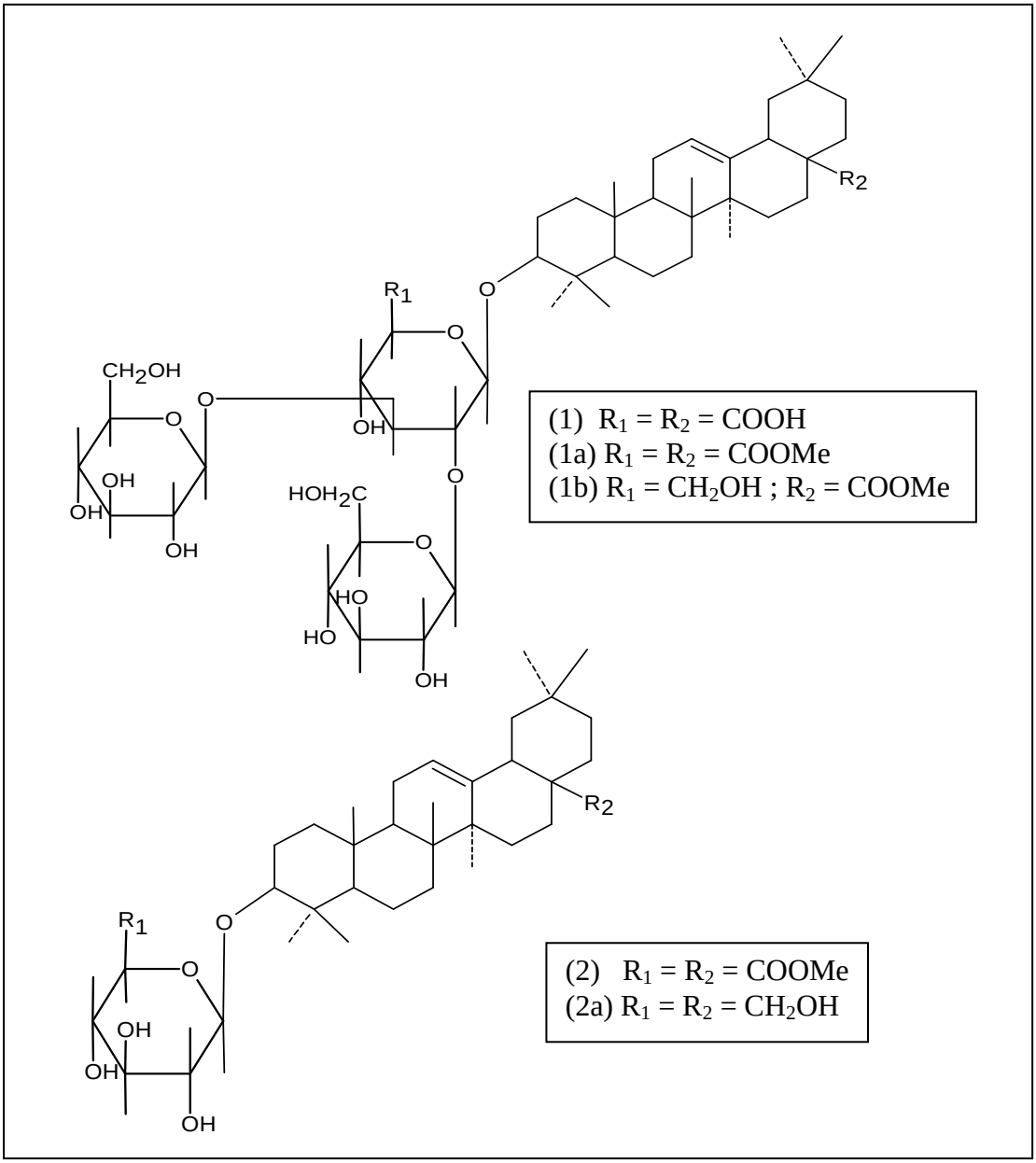


Figure 2.13: Compounds isolated from dried powdered fruits of *P. grandifolia* (Sahu *et al.*, 1974)

2.8 Bioactivity assays

Bioassay-guided fractionation is regarded as the most practical experimental approach for natural product drug discovery. In this endeavour, the plant materials are selected and extracts are prepared for biological assessment. The extracts are then subjected to a suitable bioassay system. The active extracts are identified and chose for isolation and purification of pure compound. This process continues until a pure active

substance is obtained. The resulting substance is then subjected to structure elucidation and biological assessment.

2.9 Antioxidant activity

2.9.1 Oxygen and singlet oxygen

Oxygen is an indispensable element throughout the lifetime of aerobic living organisms such as prokaryotes, fungi, eukaryotes, plants and animals. Nevertheless, oxygen is undeniably a double sword as it is also a lethal toxin for living organisms (Benzie, 2000). When we breathe oxygen in its natural state of two atoms, it poses no problems. However, approximately 1 to 3 % of the oxygen (O_2) consumed is incompletely metabolized in the mitochondria and thereby diverted into superoxide ion ($O_2^{-}\cdot$) generation. The mitochondrial respiratory chain produces ROS (reactive oxygen species) as by-products of electron transport (Ahmad, 1995).

Oxygen is easily broken down to singlet oxygen ($^1O_2$) by heat, UV and photochemical reactions of many compounds. Singlet oxygen molecules are incomplete, energetically unstable, highly reactive and short-lived. Stability is achieved by the removal of electrons (i.e. oxidation of) from surrounding molecules to claim their lost electron, thus creating other new ROS with unpaired electrons in the process. These ROS attack other molecules, leading to the propagation of a free radical chain reaction (Ramarathnam *et al*., 1995).

2.9.2 Free radicals and Reactive Oxygen Species (ROS)

A free radical is defined as any atomic or molecular species capable of independent existence that contains one or more unpaired electrons in one of its molecular orbital (Halliwell *et al*., 1992). Free radicals are also termed as ROS with one or more unpaired electrons, to describe both radical and non-radical oxidants. The

radical ROS included superoxide ($O_2^{-}\cdot$), hydroxyl (OH), nitric oxide (NO ·) and peroxyl (ROO ·). Non-radical ROS are hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($^1O_2$) and hypochlorous acid (HOCl) (Aruoma, 1994).

Free radicals are generated constantly in the body due either to physiological metabolism or pathological alterations (Gate *et al*., 1999; Benzie, 2000). When an imbalance occurs between oxidants and antioxidants in favour of oxidants, excess ROS are formed; these may contribute to the aging process as well as to chronic disease such as cancer and coronary heart disease (Ames and Shigenaga, 1992; Stocker, 1999; Liu *et al*., 2000; Benzie, 2000). Among the major cellular and extra-cellular components, proteins, enzymes, lipids, DNA and RNA form the primary targets for the ROS. However, oxidation of the unsaturated fatty acid components of cell membrane is the oxidative event that occurs most frequently inside the human body (Ramarathnam *et al*., 1995).

2.9.3 Cellular effects of oxidative stress and related diseases

Oxidative stress occurs in most human diseases (Halliwell and Gutteridge, 1999). However, this is not to say oxidative stress is the cause of most diseases. The increment of free radicals may be secondary to the disease process. Oxidative stress causes three major cellular effects: (i) lipoperoxidation (ii) DNA oxidation, and (iii) protein oxidation.

Lipid peroxidation occurs in polyunsaturated fatty acids. The peroxidation of unsaturated fatty acids can induce the conversion of several fatty acid side chains in lipid hydroperoxides, which in turn leads to the formation of a reaction chain (Gate *et al*., 1999). Previous researches indicated that lipid peroxidation increased significantly in several pathologies such as diabetes (Sato *et al*., 1979), hyperglycemia (Esterbauer *et al*., 1990), atherosclerosis (Plachta *et al*., 1992) and liver diseases (Rouach *et al*., 1997).

The cholesterol or fatty acid of the plasma low-density lipoprotein (LDL) can also be oxidized during oxidative stress. Oxidized LDL is considered to be the major factor in the development of atherosclerosis (Steinberg *et al*., 1989; Galle *et al*., 1995; Gate *et al*., 1999).

Damage to DNA strands can occur directly by ROS in close proximity to the DNA or indirectly, for example, by impairing production of protein needed to repair DNA. Alteration in DNA leads to mutagenesis and carcinogenesis (Ames, 1983; Floyd, 1990). Moreover, alteration in DNA has been suggested to be responsible in part on the processes of aging (Fraga *et al*., 1990), diabetes mellitus (Dandona *et al*., 1996), inflammatory diseases (Ames, 1983) and liver disease (Sipowicz et al., 1997). Furthermore, lipid peroxidation is also a major cause of food deterioration, affecting colour, flavour, texture and nutritional value (Halliwell and Gutteridge, 1999).

Proteins are also target for free radicals. Protein oxidation alters signal transduction mechanism, transport system or enzyme activities and triggers antibody formation and autoimmune processes (Halliwell and Gutteridge, 1999; Wiseman and Halliwell, 1996). Oxidized proteins may be also associated in atherosclerosis and aging (Berliner and Heinecke, 1996; Stadtman, 1992).

2.9.4 Biological antioxidant defence mechanisms

Excess production of ROS such as hydrogen peroxide, superoxide anion, hydroxyl and other radicals is known to damage cells. However, most living organisms have efficient defences and protective systems in the cells that are essential for defending the organism against the oxidative stress caused by ROS (Osawa, 1995). A biological antioxidant has been defined as ‘any substance that, when present at low concentrations compared to those of oxidisable substrate, significantly delays or prevents oxidation of that substrates’ (Benzie and Strain, 1999).

Antioxidants are characterized by their origins. Antioxidants that are present in human cells are of two main categories, which are enzymatic antioxidants and non-enzymatic antioxidants. Cells have developed several enzymatic antioxidant systems which convert oxidants into non-toxic molecules, thus protecting the organisms from potentially damaging effects of ROS (Gate *et al*., 1999). Enzymatic antioxidants are generated *in situ* by cellular metabolism to stabilize the reactive free radicals by converting them to more stable state. Thus the intracellular environment contains enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase.

There are a variety of non-enzymatic antioxidants and low molecular mass molecules which are important in scavenging ROS, such as Vitamin E, Vitamin C, ß-carotene, selenium, ascorbate, carotenoids and Vitamin A (Gate *et al*., 1999). Epidemiological evidence indicates that the consumption of food stuffs containing antioxidant phytonutrients, such as flavonoids and polyphenolics, is advantageous for health although the human body has defence mechanisms to combat and reduce oxidative damage (Cao *et al*, 1998; Pulido *et al*., 2000).

2.9.5 Synthetic and natural antioxidants

A number of synthetic antioxidants, such as 2- and 3-*tert*-butyl-4-methoxyphenol (i.e. butylated hydroxyanisole, BHA), 2,6-di-*tert*-butyl-4-methylphenol (i.e. butylated hydroxytoluene, BHT) and *tert*-butylhydroquinone (TBHQ) have been added to foodstuffs to prevent the oxidation of food, but their safety has been questioned due to toxicity issue (Valentao *et al*., 2002). BHA and BHT were found to be anticarcinogenic as well as carcinogenic in experimental animals. Originally, BHA appeared to have tumour-initiating as well as tumour-promoting action. However, later research reported that BHA and BHT also caused tumour promotion (Botterweck *et al*., 2000).

Therefore, there has been considerable interest in the development and isolation of natural antioxidants from botanical sources, especially edible plants to replace synthetic antioxidants. Natural antioxidants are presumed to be safe since they occur in plant foods and are seen as more desirable than their synthetic counter-parts (Amarowicz *et al*, 2004; Sakanaka *et al*., 2004). According to Pokorny (1991) when compared to synthetic antioxidants, natural antioxidants have the following advantages: (i) they are readily acceptable by the consumers (ii) they are considered to be safe (iii) no safety tests are required by legislation (iv) this natural antioxidant (not as a synthetic chemical antioxidant) is identical to the food which people have taken over a hundred years or have been mixing with food (Bera *et al*., 2006).

Unlike synthetic antioxidants, which are phenolic compounds with varying degrees of alkyl substitution, natural antioxidants can be phenolic compounds (flavonoids, phenolic acids and tannins), nitrogen-containing compounds (alkaloids, chlorophyll derivatives, amino acids, peptides and amines), carotenoids, tocopherols or ascorbic acid and its derivatives (Velioglu *et al*., 1998). The roles of these compounds as potential antioxidants can be inferred by their similarity to synthetic antioxidants, of related structures (Larson, 1988).

Flavonoids and other plant phenolics, such as phenolic acids, tannins, lignans and lignin, are especially common in the leaves, flowering tissues, and woody parts, such as stems, bark and roots of plants. The antioxidant activity of these phenolics is mainly due to their redox properties, which allow them to act as reducing agents or hydrogen-atom donors. The natural antioxidants are functioning as free radical scavengers and chain breakers, complexes of pro-oxidant metal ions and quenchers of singlet-oxygen formation (Pratt, 1992).

2.9.6 Methods to determine antioxidant activities

Ever since the epidemiological consequences of free radicals with certain diseases were disclosed, there is a growing interest in searching for the beneficial health effects of certain foods and plants (Lee and Halliwell, 2001). It is therefore of great interest to measure the antioxidant capacity using various radicals and antioxidant systems properly. Thus, convenient methods for quick and simple quantification of the antioxidant capacity are needed.

There are two major groups of commonly used methods for total antioxidant activity determination, which are (i) assays based on a single electron transfer reaction, monitored through changes in colour as the oxidant is reduced, and (ii) assays based on a hydrogen atom transfer reaction, where the antioxidant and the substrate (probe) compete for free radicals (Huang *et al*., 2005; Tabart *et al*., 2009).

Electron transfer reaction assays include the trolox equivalent antioxidant capacity (TEAC) assay, ferric reducing ability of plasma (FRAP) assay, copper reduction (CUPRAC) assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay and Folin-Ciocalteau method. Hydrogen atom transfer reaction assays include the crocin bleaching assay, total peroxyl radical-trapping antioxidant parameter (TRAP) assay and oxygen radical absorbance capacity (ORAC) assay. Other methods not included in these two groups have been developed, such as the total oxidant scavenging capacity (TOSC) assay, the chemiluminescence assay and the electrochemiluminescence assay (Huang *et al*., 2005; Prior *et al*., 2005; Tabart *et al*., 2009).

As stressed by Frankel and Meyer (2000) and Huang *et al*. (2005), no single method is adequate for evaluating the antioxidant capacity since different methods can yield widely diverging results. Tabart *et al*. (2009) suggested various methods based on

different mechanisms, must be used in parallel to evaluate the antioxidant capacity of compounds or foods, since different methods can give very different results.

2.10 Antimicrobial activity

A large number of human, animal and plant disease are caused by pathogenic microbes (fungi, bacteria and algae). Within the recent years, infections have increased to a great extent. Infections due to fungi and bacteria have been a major cause of death in higher organisms. Infectious diseases, particularly skin and mucosal infections, are common in most of the tribal inhabitants due to lack of sanitation, potable water and awareness of hygienic food habits (Ahmad and Beg, 2001).

Foodborne illness resulting from consumption of food contaminated with pathogenic bacteria has been of vital concern to public health. Foodborne illness is a serious health threat and has significant economic consequences for people in both the developing and developed world. Foodbourne illness can be defined as 'any illness of an infectious or toxic nature caused by or thought to be caused by the consumption of food or water'. The illness caused by foodborne microorganisms, principally bacteria, are associated with gastrointestinal symptoms of nausea, vomiting, stomach pains and diarrhoea. Since diarrhoea is a common clinical symptom in foodborne illness, many of these illnesses are referred to as 'diarrhoeal illnesses' (Lin *et al*., 2005).

An estimated 76 million cases occur annually in the United States, resulting in 300,000 hospitalizations and 5,000 deaths (Mead *et al*., 1999). Among the reported outbreaks in the United States during 1993-1997 periods for which the aetiology was determined, bacterial pathogens caused the largest outbreaks (75 %) and the largest percentage of cases (86 %). *Salmonella spp*., *Listeria monocytogenes* and *Escherichia coli* accounted for the largest number of breakfasts, cases and deaths (Todd, 1989).

Gram-positive bacteria are common pathogens responsible for skin disease conditions. These include *Staphylococcus aureus* for skin infections (Darmstadt and Lane, 1994; Trilla and Miro, 1995; Noble, 1998), *Staphylococus pyogenes* for pharyngitis, tonsillitis and impetigo (Cappelletty, 1998; Cunningham, 2000) and both organisms for otitis media (Brook, 1994; Brook and Gober, 1998).

The treatment of these infections is mainly based on the use of antibiotics. Historically many of the new antibiotics were isolated from natural sources (i.e. soil microbes, plants). Many more were later synthesized and introduced in clinical practices. The discovery of antibiotic penicillin by Fleming is therefore considered to be one of the most important discoveries in the world.

However, in recent years, a number of antibiotics have lost their effectiveness due to the development of resistant strains, mostly through the expression of resistance genes (Davis, 1994; Service, 1995). In addition, antibiotics are sometimes associated with adverse effects including hypersensitivity, immune-suppression and allergic reaction (Ahmad *et al*., 1998).

The discovery and development of new antimicrobial agent is therefore an ongoing process. Remarkable diversity of chemicals present in natural medicinal plant has tremendous potential in the searching of new antimicrobial agents with possibly novel mechanisms of actions (Ramzi and Ulrike, 2005). The compounds that can either inhibit the growth of pathogens or kill them and have no or least toxicity to host cells are considered candidates for developing new antimicrobial drugs. In recent years, antimicrobial properties of natural products are being increasingly reported from different parts of the world.

Disease burden, infection and foodbourne illness caused by microorganisms tested in present study

(i) Gram negative bacteria

Escherichia coli

Escherichia coli, *Shigellas*, *Salmonellas* and *Vibrio cholerae* are the common pathogens and major cause of foodbourne illness in worldwide. Diarrhoea caused by a variety of *E. coli* strains is a major problem in developing countries, especially children living in the developing world (Girard *et al*., 2006). Disease caused by *E. coli* follows ingestion of contaminated food or water and is characterized by profuse watery diarrhoea lasting for several days. It may lead to dehydration and malnutrition in young children in developing countries. Infection by *E .coli* strain O157:H7 is accompanied by a watery diarrhoea rapidly progressing to frank haemorrhagic colitis, and leads in up to 20 % of paediatric patients to serious sequelae, including the haemolytic uremic syndrome (HUS) characterized by thrombocytopenia, haemolytic anaemia and renal failure which can lead to permanent loss of kidney function (Karmali, 2004).

Pseudomonas aeruginosa

Infections caused by *P. aeruginosa* are among the most difficult to treat with conventional antibiotics (Levison and Jawetz, 1992). *P. aeruginosa* is an opportunistic pathogen that causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia and a variety of systemic infections, particularly in victims of severe burns, and in cancer and AIDS patients who are immunosuppressed (Trafny, 1998; Yau *et al*., 2001). The bacterium almost never infects uncompromised tissues, yet there is hardly any tissue that it cannot infect, if the tissue defences are compromised in some manner (Levison and Jawetz, 1992).

(ii) Gram positive bacteria

Staphylococcus aureus

S. aureus is a gram positive bacterium known to play a significant role in invasive skin diseases including superficial and deep follicular lesions. Skin and skin structure infections (SSSIs) are among the most common infectious encountered in medical practice. Skin and skin structure infections are defined by the United States Food and Drug Administration (FDA) as uncomplicated and complicated. Uncomplicated SSSIs include simple abscesses, impetiginous lesions, furuncles and cellulitis. Complicated SSSIs (cSSSIs) include deeper soft-tissue infections requiring significant surgical intervention such as infected ulcers, burns, and major abscesses or an infection occurring in the setting of a significant underlying diseases state that complicates the response to treatment (Yang and Kerdel, 2006).

Bacillus subtilis

Bacillus spp. is commonly microbes found in natural environmental including soil, plant, water and animal tissues. Most *Bacillus spp.* are regarded as having little pathogenic potential. *B. subtilis* has been known to act as invaders or secondary infectious agents in a number of diseases and has been implicated in some cases of food poisoning (Turnbull and Kramer, 1991).

2.11 *In vitro* cytotoxic activity tests

2.11.1 *In vitro* testing system

The application of *in vitro* assay systems for the search of potential chemopreventive and anticancer agents has been common since the beginnings of cancer chemotherapy in 1946 (Wilson, 2000). The use of cell culture systems basically offer numerous advantages over whole animal models (*in vivo* system), being

inexpensive, sensitive, with reproducibility of test conditions, rapid and most importantly no ethical constraints associated with using animal experimentation for screening of cytotoxic activity (Borenfreund and Puerner, 1985; Stark *et al.*, 1986; Cordell *et al.*, 1991).

In general, the *in vitro* test systems can be divided into two groups; one consisting of chemically-induced cell transformation system, which are used to detect changes in initiation versus promotion process, and the other comprising of cells with identified oncogenes or transforming growth factors. The endpoint of these assays is to observe inhibition effects of cell proliferation, transformation or differentiation (Greenwald *et al*., 1990). Table 2.1 outlines several endpoints used to identify cytotoxic effects of chemopreventive or anticancer agents using *in vitro* test system (Knox *et al*., 1986).

Table 2.1: Endpoints used to identify cytotoxic effects using *in vitro* test system (Knox *et al*., 1986)

Class	**Endpoint**
Cell viability	Vital dye uptake; dye exclusion; detachment from culture dish surface; fall in cell number; replating efficiency
Cell morphology	Cell size and shape; cell-cell contacts; nuclear size, shape and inclusions; nucleolar number, size, shape and inclusions; cytoplasmic vacuolation.
Cell proliferation	Increase in cell number, total DNA, total RNA, total protein; colony formation on culture dish surface.
Membrane damage	Loss of enzymes, ions or cofactors; leakage from prelabelled cells.
Uptake or incorporation of radioactive precursors	Uridine and RNA synthesis; thymidine and DNA synthesis; amino acids and protein synthesis
Metabolic effects	Inhibition of metabolic co-operation; cofactor depletion

2.11.2 *In vitro* cytotoxic activity testing

The discipline of cell toxicology (cytotoxic activity) is defined as quantitative and qualitative investigations of adverse effects of chemical and physical factors on cells, including toxicity ranking, extrapolation to the organism level and risk evaluation. Cell toxicology can be said to link together the disciplines of cell biology and toxicology (Walum *et al*., 1990).

It is important to distinguish between the terms 'cytotoxic', 'antitumor' and 'anticancer'. 'Cytotoxic' compounds are toxic to cells in culture and maybe cytostatic (*i.e*. stop cell growth, reversibly or irreversibly) or cytocidal (kill cells). 'Antitumor' compounds are those which are active in an *in vivo* tumour system. Such compounds would therefore show selectivity against tumour cells. 'Anticancer' compounds are those which are effective in cancers in humans. Hence it requires human clinical trials to determine if any antitumor compound has anticancer activity (Rahman *et al*., 2001).

In vitro cytotoxicity assays can be used to predict human toxicity and for the general screenings of chemicals (Clemedson and Ekwall, 1999; Scheers *et al*., 2001). Cytotoxicity screening models provide important preliminary data to help select plant extracts with potential antineoplastic properties for future work (Cardellina *et al*., 1999). In past years, a number of methods have been developed to study cell viability and proliferation in cell culture (Cook and Mitchell, 1989). The most convenient, modern assays have been optimized for the use of microtiterplates (96- well format). Each well requires only a small amount of test sample so that several replicates can be performed and a range of concentrations tested. This allows many samples to be analyzed rapidly and simultaneously. Colorimetric and luminescence based assays allow samples to be measured directly in the plate by using a microtiterplate reader or ELISA plate reader. Cytotoxicity assays have been developed which use different parameters associated with cell death and proliferation (Weyermann *et al*., 2005).

2.11.3 Methods to determine cytotoxic activity

Cytotoxicity assays are widely used in *in vitro* toxicology studies. It has been previously reported that different cytotoxicity assays can give different results depending on the test agent used and the cytotoxicity assay employed. For this reason, it is important to consider what effect is expected, respective of what cell death mechanism is predicted when choosing a suitable cytotoxicity assay for the studies (Weyermann *et al*., 2005).

The lactate dehydrogenase leakage (LDH) assay, a protein assay, the neutral red assay, the methyl tetrazolium (MTT) assay and ATP content assay are the most common employed assays for the detection of cytotoxicity or cell viability following exposure to toxic substances (Fotakis and Timbrell, 2006; Weyermann *et al*., 2005). These assays are based on different modes of detection like LDH release which is based on the release of enzyme into the culture medium after cell membrane damage; protein content of viable cells that are left after washing of the treated plates; neutral red uptake by functional lysosomes; MTT metabolism which is based on the enzymatic conversion of MTT in mitochondria and ATP content of treated cells (Fotakis and Timbrell, 2006). Thus, one has to be careful with the suitability of the assay used to prevent false-positive or false-negative results.

2.12 Apoptosis screening and detection

There are several typical methods for apoptosis screening and detection (Table 2.2). However, each method to detect and verify apoptosis has advantages and disadvantages. It is suggested to use multiple complementary techniques to confirm apoptosis.

Table 2.2: Typical methods to study different aspect of apoptosis (Huerta *et al.*, 2007)

Detection aspects	Methods / assay
Morphological changes	• Microscopic techniques ❖ Electron microscopy (EM) ❖ Light microscopy (LM) ❖ Fluorescence microscopy (FM) • DNA staining assay ❖ Dye exclusion ❖ DNA binding dyes ❖ Annexin V
DNA fragmentation	• TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling) • ISEL (*In situ* end labelling technique) • FIGE (Field inversion gel electrophoresis) • Flow cytometry and ELISA (enzyme-linked immunosorbent assay) • DNA laddering • DNA fragmentation measurement • APO ssDNA

2.12.1 Detection of morphological changes

Microscopic techniques

One of the methods to detect apoptosis by observation of morphological changes is microscopic techniques, which include electron microscopy (EM), light microscopy (LM) and fluorescence microscopy (FM). EM is time consuming and requires expensive equipment and specialized training (White and Cinti, 2004) while LM and FM can only be used to detect the late events of apoptosis (Huerta *et al.*, 2007). All these microscopic techniques can detect apoptosis *in vitro* but are limited by the fact that they can only detect apoptosis at a single point in time and inability to screen a

large number of samples. Thus, it is possible to miss the characteristic apoptosis bodies by microscopy to establish apoptosis.

DNA staining assay

Dye exclusion, DNA binding dyes and Annexin V are some of the techniques for DNA staining assays. Generally, the cell membrane of dead cells becomes permeable to dyes and can be stained. In contrast, viable cells can be excluded by dyes that will not penetrate the intact plasma membrane. Trypan blue is a typical exclusion dye that can be used to differentiate viable and dead cells by light microscopy such that blue-stained cells can be distinguished from the viable non-stained cells. In the present study, the density of the viable cells in neutral red cytotoxicity assay was counted by 0.4 % of trypan blue exclusion in a haemocytometer.

The fluorescent dyes, such as propidium iodide (PI), Hoechst 33342, 4',6-diamidino-2-phenylindole (DAPI) and YOPRO-1, become highly fluorescent upon binding of DNA in viable cells. These DNA markers make the chromatin condensation readily visible by fluorescence microscopy and simplify the detection of morphological changes during apoptosis. Thus, this is not a very specific test for apoptosis, but this can be compensated by using other detection methods.

2.12.2 Detection of DNA fragmentation

DNA fragmentation is one hallmark of apoptosis. The morphological changes observed in the nucleus of apoptotic cells are, in part, due to the generation of DNA fragments through the action of endogenous endonucleases, including the caspase-dependent DNase (Arends *et al*., 1990; Gavrieli *et al*., 1992). Typically, the DNA of apoptotic cells is cleaved to a population of multimers of 180-200bp fragments, readily observed as a 'ladder' on agarose gels.

There are several established assays that are used for the detection of DNA fragmentation as listed in Table 2.2 and TUNEL [terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling] assay is the most widely used *in situ* test among all the techniques.

2.12.3 TUNEL assay

The TUNEL assay was introduced by Gavrieli *et al*. (1992). TUNEL assay is based on the specific binding of terminal deoxynuclotidyl transferase (TdT) to 3'-OH ends of fragmented DNA (nicks). Following proteolytic treatment of histological sections, TdT incorporates X-dUTP (X=biotin, DIG or fluorescein) at sites of DNA breaks. Termini modified nucleotides Avidin-peroxidase amplifies the signal and allows for examination of labelled cells under light or fluorescent microscopy, flow cytometry or immunohistochemistry (Gavrieli *et al*., 1992).

Limitations of TUNEL assay

The sensitivity and specificity of the TUNEL assay has been criticized, and it has been reported that several factors can produce a false-positive signal in TUNEL assay (Pulkkanen *et al*., 2000). The following are some limitations of the TUNEL assay:

i. Fixation and handling of cells can significantly alter the results of TUNEL assay (Charriaut-Marlangue and Ben Ari, 1995; Ichimura *et al*., 1995; Lucassen *et al*., 1995).
ii. The rates of apoptosis might be inaccurate if fixation is prolonged (Davison *et al*., 1995).
iii. Measurement of apoptosis at a single time point in cell or tissue sections may be an inaccurate representation of the true rate of programmed cell death.

iv. TUNEL assay is not specific for apoptosis. It indicates DNA cleavage from any form of cell death and necrotic cells may also be labeled by this technique (Grasl-Kraupp *et al*., 1995).

Hence, TUNEL is an accepted assay to establish apoptosis *in vitro* and *in situ* and when confirmed with other methods, it is a reliable test for apoptosis (Huerta *et al.*, 2007). It is advisable to employ two independent methods (e.g. DNA fragmentation and caspase-3 activation) to confirm that cell death is occurring by apoptosis. Thus, the detection of DNA fragmentation by using DeadEnd^{TM} Colorimetric TUNEL System had been carried out with determination of expression level of apoptotic related genes in the present study.

2.13 Determination of expression level of apoptotic-related genes

The therapeutic application of apoptosis is currently being considered as a model for the development of anti-tumour drugs (Hong *et al.*, 2003). It is therefore essential to identify apoptosis-inducing compounds that are candidate anti-cancer agent. Thus, the importance of investigating the role of gene expression in the identification of potential chemotherapeutic agents using mechanism-based studies holds great promise for elucidating mechanisms and devising more specific and effective treatments for cancer-related diseases.

2.13.1 Introduction to gene expression

Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product. There are several steps in the gene expression process, including the transcription step and translation step. Genes are expressed by being transcribed into RNA, and this transcript may then be translated into protein (Figure 2.14).

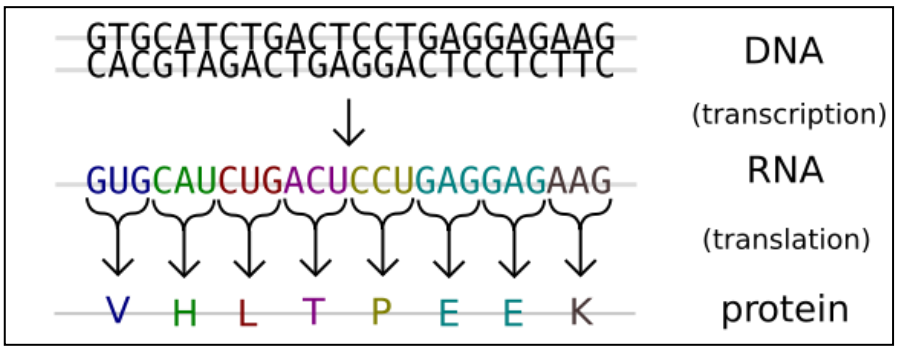


Figure 2.14: Steps in gene expression process (Adapted from http://en.wikipedia.org/wiki/Gene_expression, 20 April 2009)

The gene itself is typically a long stretch of DNA and does not perform an active role. It is a blue print for the production of RNA. The production of RNA copies of the DNA is called transcription and is performed by RNA polymerase. RNA is central to the synthesis of proteins while mRNA is the RNA that carries information from DNA (protein sequence) to the ribosome (the protein synthesis [translation] in the cell). The coding sequence of the mRNA determines the amino acid sequence in the protein that is produced. Non-protein coding genes (e.g. rRNA genes, tRNA genes) are transcribed and involved in the process of translation, but not translated into protein. The mRNA is then exported from the nucleus to the cytoplasm, where it is bound to the ribosome and translated into its corresponding protein form with the help of tRNA. While the genes of an organism are relatively fixed, the mRNA population represents how genes are expressed under any set of conditions.

2.13.2 Importance of gene expression measurement

Understanding the process of cell death associated with numerous disease conditions is essential in resolving the pathogenesis and identifying possible therapeutic approaches. For many years, the cytotoxic actions of the chemotherapeutic drugs were ascribed solely to their ability to induce genotoxic death (Kamesaki, 1998). However, there were accumulating evidences that these agents exert their cytotoxic effects mainly

by inducing apoptosis in tumour cells. Impairment of apoptosis is known to be related to cell immortality and carcinogenesis; the induction of apoptosis in neoplastic cells, therefore, is vital in cancer treatment.

The chemotherapeutic drugs that have been observed to induce apoptosis *in vitro* include etoposide, camptothecin, VM26, vincristine, *cis*-platinum, cyclophosphamide, paclitaxel, 5-fluorouracil and doxorubicin (Kaufman, 1989; Walker *et al*., 1991; Shinomiya *et al*., 1994; Havrilesky *et al*., 1995; Huschtscha *et al*., 1996). In accordance with these *in vitro* studies, other studies also provide evidences that chemotherapeutic agents induce apoptotic tumour cell death *in vivo*. Experimental studies of murine tumours have demonstrated that *cis*-platinum, cyclophosphamide and other chemotherapeutic agents induced apoptosis in various tumours *in vivo* (Meyn *et al*., 1995). Several clinical studies have also shown that chemotherapy triggers apoptotic cell death in patients undergoing chemotherapy (Gorczyca *et al*., 1993; Moreira *et al*., 1995).

Therefore, it is important to determine the expression level of apoptotic-related genes in order to determine the possible mechanisms of cell death elicited by the active extracts and isolated compounds on the selected cell lines.

2.13.3 Apoptotic-related genes

Apoptosis is a complex process that involves a variety of different signalling pathways that lead to a multitude of changes in the dying cells. Three apoptotic-related genes used in the present study were p53, caspase-3 and c-myc.

p53

The p53 is the first tumour suppression gene linked to apoptosis which is expressed in a variety of malignancies (Kaabinejadian *et al*., 2008). Tumour

suppression gene is a gene that protects a cell from becoming cancer one. p53 has appeared as a key tumour suppressor and important target for novel cancer therapy. It plays a major role in preventing tumour development (Mills, 2006).

Cellular tumour antigen p53 is a transcription factor with multiple functions including regulation of the cell cycle, inhibition of angiogenesis, DNA repair and apoptosis (Lacroix *et al*., 2006). p53 is a nuclear protein and also known as tumour protein 53 (TP53), tumour suppressor p53, phosphoprotein p53 and antigen NY-CO-13. It has been described as 'the guardian of the genome', which referred to its role in conserving stability by prevent genome mutation and selectively inhibit the growth of eradicate damaged cells (Lane, 1992). p53 plays an important role in cellular responses to a variety of environmental and intracellular stresses including DNA damage, UV radiation, hypoxia and hyper-proliferation (Vousden and Lu, 2002; Meek, 2004). It has been implicated as an important determinant of tumour cell sensitivity to cytotoxic agents (Kaabinejadian *et al*., 2008). Tumours that lack p53 function often respond poorly to radiation and chemotherapy (Michalak *et al*., 2005).

The p53 signalling pathway is in standby mode under normal cellular conditions. Activation occurs in response to cellular stresses and leads to an increase in the level of p53 protein due to reduced MDM2-dependant proteolytic degradation and increased affinity of p53 for DNA. Since p53 exerts its anti-proliferative action by inducing cell cycle arrest or apoptosis, loss of p53 activity, which often occurs in cancer, disrupts apoptosis and accelerates the appearance of tumours (Kaabinejadian *et al*., 2008).

Caspase-3

Caspase-3 was used as one of the apoptotic markers in the present study. Many of the events that occur during apoptosis are mediated by caspases, a family of cysteine proteases (Alnemri *et al*., 1996; Cardone *et al*., 1988; Chen *et al*., 2001). The family of

caspases formed an executionary arm that implements the apoptotic cell death processes. Caspases are normally present in the cell as proenzymes that require limited proteolysis for activation of enzymatic activity (Nunez *et al*., 1998). It was well documented that once activated, caspases cleave a variety of intracellular polypeptides, including major structural elements of the cytoplasm and nucleus, components of the DNA repair machinery and a number of protein kinases. Collectively, these scissions disrupt survival pathways and disassemble important architectural components of the cells, contributing to the stereotypic morphological and biochemical changes that characterize apoptosis cell death (Earnshaw *et al*., 1999).

Caspases are divided into pro-apoptotic (caspase 2, 3, 6, 8, 9 and 10) and pro-inflammatory (caspase 1, 4 and 5) members. Among the caspases, caspase-3, which is recognized as a central player in mediating cellular apoptosis, is the most commonly activated caspases in the apoptosis process (Janicke *et al*., 1998). The activation of caspase-3 is an important downstream event in apoptosis. Caspase-3 has been widely shown to mediate the limited proteolysis of the structural protein gelsolin, p21-activated kinase 2 (PAK2), focal adhesion kinase (FAK) and rabaptin 5 (Cosulich *et al*., 1997; Kothakota *et al*., 1997; Rudel and Bokoch, 1997; Wen *et al*., 1997) and cleavage inactivation of DNA fragmentation factor such as DFF45 and ICAD (Liu *et al*., 1997; Enari *et al*., 1998; Sakahira *et al*., 1998). Evidences of caspase 3 involvements in the apoptosis process are well documented, for example, caspase-$3^{-/-}$ mice (caspase-3-knockout mice) can survive to birth, but they exhibit perinatal mortality as a result of defects in brain development that correlate with a decrease in levels of apoptosis (Kuida *et al*., 1996; Woo *et al*., 1998). Caspase-3 was also reported to be required for apoptosis in neutrophils and activated T-cells (Woo *et al*., 1998).

C-myc

The c-myc gene is the main member of Myc-family that also includes N-myc, L-myc, S-myc and B-myc. Like other Myc proteins, c-myc is important in organization, with the crucial regions needed for proliferation, apoptosis and transcriptional activities located in its terminal domains (Prendergast, 1999). It is a regulator of cell cycle progression and can cause cells to undergo apoptosis (Askew *et al*., 1991; Evan *et al*., 1992). C-myc is also an enhancer of apoptosis (McCarthy *et al*., 1994), which was shown to enhance activation-induced cell death in T-cell hybridomas (Shi *et al*., 1992). An increased expression of c-myc has been demonstrated to lead cells into apoptotic route (Wurm *et al*., 1986; Wyllie *et al*., 1987; Askew *et al*., 1991; Evan *et al*., 1992).

2.13.4 Methods of mRNA quantification

There are four methods in common use for the quantification of mRNA: (i) northern blotting (Parker and Barnes, 1999), (ii) ribonuclease protection assay (Hod, 1992; Saccomanno *et al*., 1992), (iii) *in situ* hybridization (Parker and Barnes, 1999) and (iv) competitive reverse transcriptation-polymerase chain reaction (RT-PCR) (Weis *et al*., 1992). These methods are time consuming and require considerable effort.

Northern analysis is the only method providing information about mRNA size, alternative splicing and the integrity of RNA samples. The ribonuclease protection assay is most useful for mapping transcript initiation and termination sites and intron/exon boundaries, and for discriminating between related mRNA of similar size, which would migrate at similar positions on a northern blot. There is no amplification involved in northern blotting and ribonuclease protection assay. *In situ* hybridization is the most complex method of all, but is the only one that allows localization of transcripts to specific cells within a tissue (Bustin, 2000). Techniques such as Northern blotting and ribonuclease protection assays work very well, but time consuming and

require more RNA than is sometimes available. Thus, the main limitation of these three techniques is their comparatively low sensitivity (Melton *et al*., 1984).

RT-PCR is an *in vitro* method for enzymatically amplifying defined sequences of RNA (Rappolee *et al*., 1988) and permits the analysis of different samples when amounts of RNA are low. It is the most sensitive and the most flexible of the quantification methods (Wang and Brown, 1999) and can be used to compare the levels of mRNAs in different sample populations, to characterize patterns of mRNA expression, to discriminate between closely related mRNAs, and to analyse RNA structure (Bustin, 2000).

2.13.5 RT-PCR quantification

It is important to review the nature of the PCR in order to understand RT-PCR quantification. The advantages of PCR, accounting for its success in gene expression, have been its sensitivity, specificity and rapidity. PCR is conceptually simple and amenable to full automation. PCR techniques allow for the *in vitro* synthesis of millions of copies of a specific gene segment of interest, allowing the rapid detection of as few as 1 to 10 copies of target DNA from the original sample (Figure 2.15) (DeBiasi and Tyler, 2004).

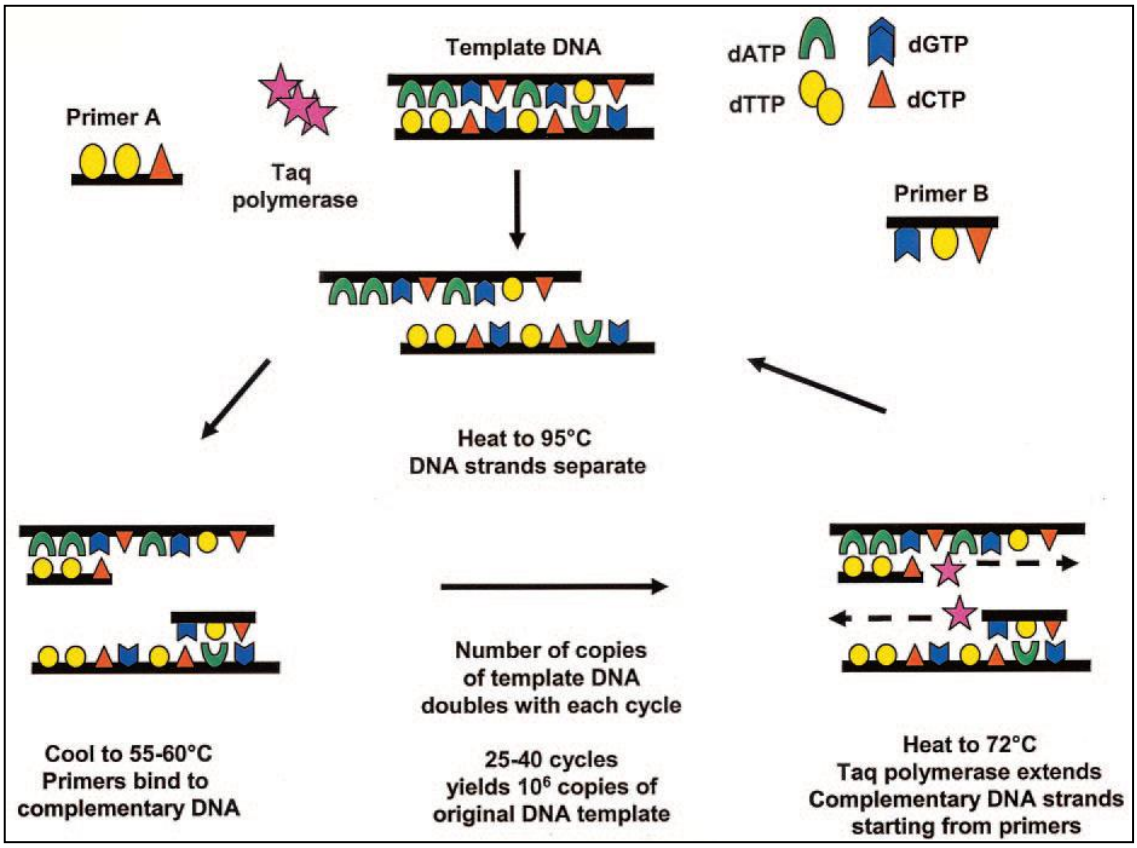


Figure 2.15: General PCR schema (DeBiasi and Tyler, 2004)

(i) Conventional RT-PCR quantification

Conventional RT-PCR is a complex assay (Figure 2.16A) and all physical and chemical components of the reaction are interdependent. The conventional RT-PCR assay measures the end-product of PCR amplification after a given number of cycles and involves some methods for quantitating the bands on the gel (end-point quantitative RT-PCR) (Yuan *et al*., 2000). Conventional RT-PCR is only semi-quantitative at best, in part because of the insensitivity of ethidium bromide stain and also the difficulties of observing the reaction during the truly linear part of the amplification process and get truly quantitative results. Various competitive PCR protocols have been designed to overcome this problem but they tend to be cumbersome and time consuming to perform. DNA sequencing may be required to confirm the identity of the amplicon. Thus, real-time RT-PCR (RT-qPCR) has been developed so that more accurate results can be obtained to quantitate differences in mRNA expression.

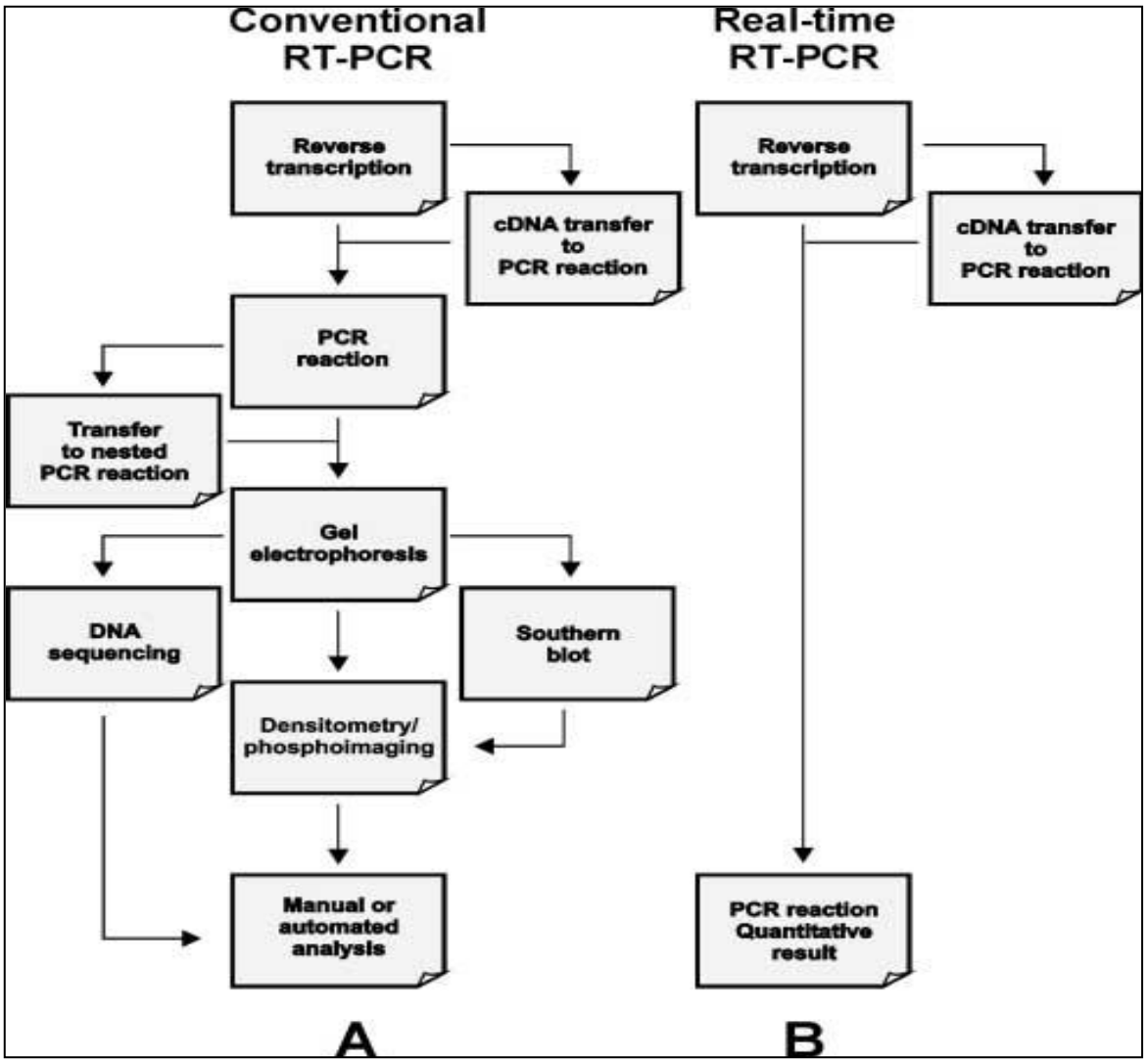


Figure 2.16: RT-PCR quantification
(A) Conventional RT-PCR (B) Real-time RT-PCR (RT-qPCR) (Bustin, 2000)

(ii) The RT-qPCR quantification

Real-time PCR (qPCR) uses fluorescent reporter dyes to combine the amplification and detection steps of the PCR reaction in a single tube format (Higuchi *et al*., 1992; Higuchi *et al*., 1993). The real-time reverse transcription-polymerase chain reaction (RT-qPCR) involves of three steps: (i) the reverse transcriptase (RT)-dependent conversion of RNA into cDNA, (ii) the amplification of the cDNA using the PCR and (iii) the detection and quantification of amplified fluorescence products in real-time (Nolan *et al*., 2006). RT-qPCR schema is shown in Figure 2.16B.

qPCR is proven to be a sensitive, reliable and convenient alternative for quantifying the changes in expression of multiple genes (Bustin, 2000; Heid *et al*., 1996; Freeman *et al*., 1999). At the start of a PCR reaction, reagents are in excess while template and product are at low enough concentrations. This is to prevent product re-naturation compete with primer-binding and allows amplification to proceed at a constant and exponential rate. The amount of DNA theoretically doubles with every cycle of PCR. After certain cycles, the reaction rate ceases to be exponential and enters a linear phase of amplification, which primarily due to product re-naturation competing with primer binding. However, the reaction cannot go on forever. The amplification rate eventually drops to near zero and reaches a plateau phase with little product is made (Figure 2.17).

The amplification curve of qPCR is shown in Figure 2.17. The assay relies on measuring the increase in fluorescent signal, which is proportional to the amount of DNA produced during each PCR cycle and so the progress of the reaction can be viewed. Furthermore, the use of probes labelled with different reporter dyes allows the detection and quantification of multiple target genes in a single (multiplex) reaction. Individual reactions are characterized by the PCR cycle number at which fluorescence first rises above a defined or threshold background fluorescence, a parameter known as the threshold cycle (C_t) or crossing point (C_p). A fixed fluorescence threshold is set significantly above the baseline by the operator. The higher the starting copy number of the nucleic acid target, the sooner the significant increase in fluorescence is observed and the lower the C_t. This correlation between fluorescence and amount of amplified product permits accurate quantification of target molecules over a wide dynamic range, while retaining the sensitivity and specificity of conventional end-point PCR assays. The closed-tube (homogenous) format eliminates the need for post-amplification

manipulation and significantly reduces hands-on time and the risk of carry-over contamination (Nolan *et al*., 2006).

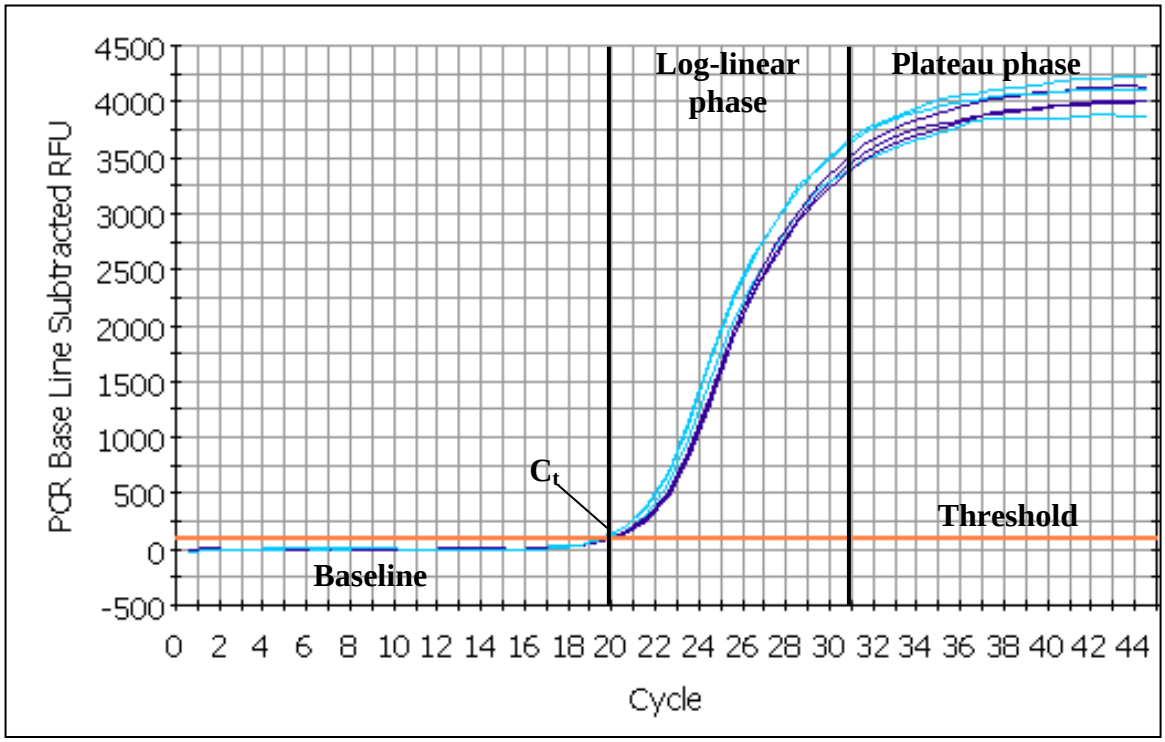


Figure 2.17: The qPCR amplification curve. A typical qPCR amplification curve is a sigmoid curve which defines by baseline, log-linear phase and plateau phase. The parameter C_t (threshold cycle) is defined as the cycle number at which the fluorescence emission exceeds the fixed threshold.

Several factors have contributed to the transformation of RT-qPCR technology into a mainstream research tool: (i) as a closed-tube format (homogenous) assay, it avoids the need for post-PCR processing (i.e. agarose gel electrophoresis) which can be time consuming and does not quantify the amount of nucleic acid within the samples, (ii) it offers a much wider dynamic range of up to > 10^7-fold (compared to 10^3-fold in conventional RT-PCR) and (iii) the assay realizes the inherent quantitative potential of the PCR, making it a quantitative as well as qualitative assay (Ginzinger, 2002).

Chemistry options for real-time amplification

There are three chemistry options for real-time amplification, which are (i) dsDNA (double-stranded DNA) intercalator dye based, (ii) probe based (i.e. hybridization probes and hydrolysis probes) and (iii) primer based. The dsDNA intercalator dye based techniques involves detection of the binding of a fluorescent dye (e.g. SYBR Green) to DNA (Morrison *et al*., 1998). TaqMan probe is a typical hydrolysis probe (Heid *et al*., 1996) which utilized the 5' exonuclease activity of Taq polymerase while hybridization probes include molecular beacons (Abravaya *et al*., 2003) and scorpions (Solinas *et al*., 2001; Terry *et al*., 2002). In present study, fluorogenic LUX primer, which is one of the primer-based chemistries, is utilized for RT-qPCR quantification. This fluorogenic LUX primer method, however, has some advantages over some methods that include the ease of design and synthesis of the fluorogenic primers (Lowe *et al*., 2003).

2.13.6 Fluorogenic LUX primer RT-qPCR assay

The use of LUX (Light Upon eXtension) primers for detection and quantification of genes on real-time platforms represents the most recent advance in real-time design (Sharkey *et al.*, 2004). This technology was established in 2002 by scientists in the Invitrogen Corporation. LUX primers are easy to use, highly sensitive and efficient for performing qPCR and RT-qPCR to quantify 100 or fewer copies of a target gene in as little as 1 pg of template DNA or RNA. This detection format includes one single-labeled, self-quenched primer and an unlabeled counterpart (Rekhviashvili *et al*., 2006). The fluorogenic LUX primer is designed to be 'self-quenched' until it is incorporated into a double-stranded PCR product, whereupon its fluorescence increases, i.e., is 'dequenched'. The counterpart PCR primer used in conjunction with the fluorogenic primer is a standard, unlabeled oligonucleotide (Lowe *et al*., 2003).

As such primers are at an early stage of development, little on them is available. They do, however, appear to represent a more economical option than other detection chemistries that are relatively easy to design (Sharkey *et al*., 2004).

LUX primer reaction

Changes in emission of fluorescence are affected by the primary and secondary structure of a LUX primer. The 3'-end of a LUX primer requires guanosine bases near the conjugated tag. The 5'-end of this fluorogenic primer is modified by the addition of a short sequence that is complementary to the labeled 3'-end of the primer. This 5' tail enables the LUX primer to assume a hairpin configuration at temperature below the melting point of the hairpin, which causes a fluorescence-quenching effect (Figure 2.18). During PCR, when a fluorogenic LUX primer binds to the complementary sequence and becomes linear, the fluorophore is dequenched and the levels of fluorescence increase by 10-fold (Figure 2.18) (Nazarenko *et al*., 2002a; Nazarenko *et al*., 2002b; Chen *et al*., 2004; Lowe *et al*., 2003; Sharkey *et al*., 2004).

The above characteristics and other standard characteristics of the primers, such as length and Tm, are included in the primer design by proprietary software called LUX Designer (Invitrogen, http:// www.invitrogen.com/lux). These design rules enable the software to output numerous primer pairs that are located throughout the target (input) sequence.

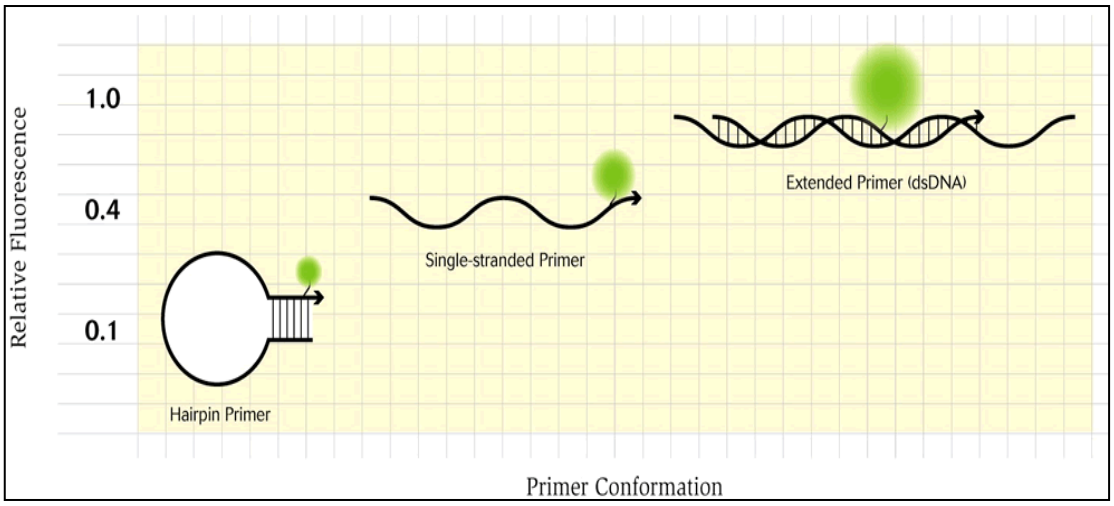


Figure 2.18: LUX primer reaction (www.invitrogen.com/lux)

Labelling

Each fluorogenic LUX primer is labelled with one of two reporter dyes, either FAM (6-carboxy-fluorescein) or JOE (6-carboxy-4',5'-dichloro-2',7'-dimethoxy-flurescein). Typically, a FAM-labelled primer set is used to detect gene of interest while a JOE-labelled primer set is used to detect a housekeeping gene as an internal control.

Melting curve analysis

Melting curve analysis during qPCR is important to identify the presence of primer dimmers and analyze the specificity of the reaction. Thus, it is encouraged to program the real-time instrument to perform melting curve analysis after thermo cycling, in which the temperature is raised by a fraction of a degree and the change in fluorescence is measured.

Melting curve analysis identifies the change in fluorescent signal that occurs when dsDNA dissociates or 'melts' into ssDNA (single-stranded DNA). The software plots the rate of change of the relative fluorescence units (RFU) with time (T) [-

d(RFU)/dT] on the Y-axis versus the temperature on the X-axis, and this will peak at the melting temperature (Tm) (Figure 2.19).

Melt curves are a powerful means of providing accurate identification of amplified products and distinguishing them from primer dimmers and other small amplification artefacts. The Tm of a DNA molecule depends on both its size and its nucleotide composition; hence GC-rich amplicons have a higher Tm than those having abundance of AT base pairs (Nolan *et al*., 2006).

All amplicon for a particular primer pair should have the same melting temperature, unless there are contamination, mispriming or primer dimmers artifacts. The presence of primer dimmers in samples containing template decreases PCR efficiency and obscures analysis and determination of C_t (threshold cycle). By identifying the temperature at which the dsDNA dissociates, small artifacts such as primer dimmers with lower annealing temperature can be distinguished from larger amplicon with higher annealing temperature.

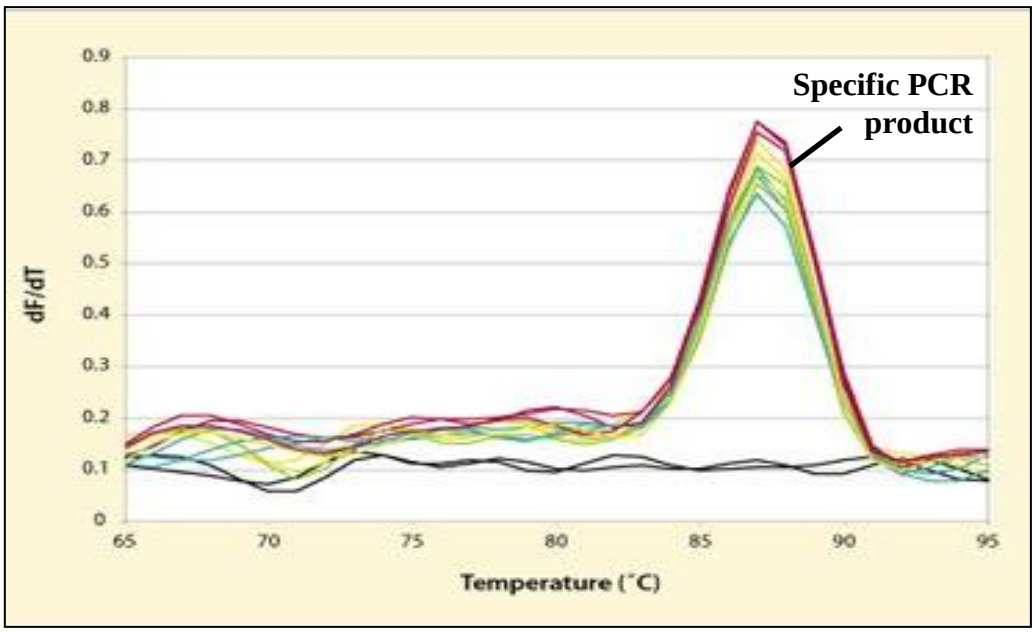


Figure 2.19: Melting curve analysis confirming specific amplification with LUX primers. The melting curve shows the changing rate of relative fluorescence [-d(RFU)/dT] over temperature (℃). qPCR was performed on 10-fold serial dilutions (10^6 to 10^3 copies) of human ß-actin plasmid using 200 nM FAM-labeled LUX™ Primer. Reactions were amplified by 50 cycles of PCR using a Corbett Research Rotor-Gene™. Melting curve analysis was performed after amplification using a temperature ramp of 1 ℃/5 sec. between 65 ℃ and 95 ℃. Colored lines indicate template-specific reactions. Black lines indicate no-template controls. Figure adapted from www.invitrogen.com/lux.

2.14 Acute oral toxicity assessment

The acute oral toxicity test is the simplest, and often the first toxicity test to be conducted on a sample. A single, high dose of the test sample is given to each experimental animal and the mortality is observed; death within the observation period (usually of 14 days duration) whether caused by natural death or humane killing is studied (Stallard, 2006).

Acute oral toxicity testing is required prior to clinical trials and results in relevant information for: (i) basic characterization of the toxic potential, (ii) early selection of suitable pharmacological substances, (iii) dose range finding, (iv) first evidence on target organs (Ukelis *et al*., 2008). Although the result does not explain the exact mechanism of toxicity observed, it does provide vital parameters for subsequent non-clinical and clinical studies.

Animal models have overall a good predictability for human toxicities of around 70-80 %. However, they are still only a model and can lead to misleading results and not predictive of human toxicity in particular if species-species biotransformation determines toxicity (Kola and Landis, 2004; Olson *et al*., 2000).

Toxic manifestations that affect the entire organism such as pain, distress, vital functions (cardiovascular, central nervous and respiratory system), allergic reactions, changes of outer appearance, behavioral alterations and general stimulation or sedation can be detected by *in vivo* assays. However, detection of effects on vital functions is usually not assessed in acute toxicity studies. Generally, it is possible to get first hints on these complex toxicities by applying *in vivo* methods, whereas information on vital functions cannot be assessed by *in vitro* cytotoxicity methods (Ukelis *et al*., 2008).

The findings of this study corroborated the need for safety study on both *Pereskia spp.* used for primary health care in Malaysia. Such studies need to be carried out before continued widespread use of some species provokes long term and irreversible damages.

LD_{50}

Traditionally, the aim of the acute oral toxicity study has been the estimation of the LD_{50}. The LD_{50} value, defined as the statistically derived dose that, when administered in an acute toxicity test, is expected to cause death in 50 % of the treated animals in a given period, is currently the basis for toxicologic classification of chemicals. For a classical LD_{50} study, laboratory mice and rats are the species typically selected. Often both sexes must be used for regulatory purposes (Walum, 1998).

Under the globally harmonized system of classification (GHS) (OECD, 1998), substances are assigned to classes 1-5, with substances in class 1 being the most toxic, according to their estimated LD_{50} values as shown in Table 2.3. Substances in class 5

are sometimes termed u*nclassified*. The classification given affects restrictions on transportation and use of the substances, as well as how it must be labelled. Substances assigned to classes 1 and 2 are labelled *fatal if swallowed*, substances assigned to class 3 are labelled *toxic if swallowed* and substances assigned to class 4 are labelled *harmful if swallowed*.

Table 2.3: GHS acute oral toxicity classifications

Class	Range for estimated LD_{50} (mg/kg)
1	$LD_{50} \leq 5$
2	$5 < LD_{50} \leq 50$
3	$50 < LD_{50} \leq 300$
4	$300 < LD_{50} \leq 2000$
5	$2000 < LD_{50}$

The absolute LD_{50} value for a compound varies among different laboratories, and these variations have been attributed to differences in e.g. protocols details, animal strains, caging and test-chemical source (Walum, 1998). In particular, the necessity to determine a high statistical accuracy in the LD_{50} value has been questioned. Oliver (1986) made the points that (i) the LD_{50} value is not an absolute value but is an inherently variable biologic parameter that cannot be described in terms of accuracy but only of precision, (ii) the LD_{50} value refers only to mortality and is illustrative of no other clinical expression of toxicity.

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant materials

The fresh leaves of *P. bleo* and *P. grandifolia* were collected from Petaling Jaya, Selangor, Malaysia in September 2006 and February 2007 respectively. They were identified by Professor Dr. Halijah Ibrahim of Institute of Biological Sciences, Faculty of Science, University of Malaya, Malaysia and deposited in the herbarium of the Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia with voucher numbers of SN01-06 (*P. bleo*) and SN01-07 (*P. grandifolia*).

3.2 Extraction and fractionation of methanol extract of plant samples

The fresh leaves of plant samples were washed, dried and ground using a blender to a fine powder. The dried, ground leaves were then soaked in methanol (1.5 L) for 3 days at room temperature. The solvent-containing extract was then decantered, dried with anhydrous sodium sulphate and filtered. The extraction of the ground leaves was further repeated (2x) with methanol (1.5 L each time). The filtrate from each extraction was combined and the excess solvent was evaporated under reduced pressure using a rotary evaporator to give a dark green crude methanol extract. Part of the methanol extract was further extracted with hexane to give a hexane-soluble extract and a hexane insoluble residue. The hexane-insoluble residue was further partitioned between ethyl acetate–water (1:1, 100 ml: 100 ml) to give an ethyl acetate-soluble

extract. The water layer was then freeze-dried to give a brown coloured water extract. The crude methanol extract and resulting extracts were then evaluated for their biological activities. The concentrations of extracts were prepared according to the requirements for each assay.

3.3 Antioxidant activity

3.3.1 Folin-Ciocalteu method

The reducing capacity of *P. bleo* and *P. grandifolia* extracts, expressed as gallic acid equivalents (GAEs), were measured according to the Folin-Ciocalteu method (Cheung *et al*., 2003; Singleton and Rossi, 1965).

Preparation of gallic acid calibration plot

A calibration plot, using gallic acid with concentrations ranging from 25 to 1000 mg/l was prepared. Gallic acid stock solutions in volumes ranging from 0.005 ml to 0.2 ml were pipetted out into test tubes. The final volume was made to 1 ml with methanol in each test tube. 0.02 ml of different concentrations of gallic acid solution and negative control (methanol was used instead of gallic acid) were mixed with 1.58 ml of distilled water. 0.1 ml of Folin-Ciocalteu's phenol reagent was added to each test tube. After 3 min, 0.3 ml of saturated sodium carbonate (Na_2CO_3) solution (~35 %) was added to the mixture. The reaction mixtures were incubated at 40 ℃ for 30 min. The blank contained only methanol. The absorbance was determined at 765 nm with a spectrophotometer. The gallic acid calibration plot was obtained by plotting the

absorbance against concentration of gallic acid (mg/l). Table 3.1 summarizes the preparation of different concentrations of gallic acid solution for calibration plot.

Table 3.1: The preparation of different concentrations of gallic acid solution for calibration plot

Gallic acid (mg/l)	Gallic acid stock solution (ml)	Methanol (ml)
0	0.000	1.000
25	0.005	0.995
50	0.010	0.990
75	0.015	0.985
100	0.020	0.980
150	0.030	0.970
200	0.040	0.960
250	0.050	0.950
500	0.100	0.900
1000	0.200	0.800

Determination of reducing capacity in test extracts

A stock extract in methanol was prepared at a concentration of 20 mg/ml. 0.02 ml of extract with different concentrations (4, 8, 12, 16 and 20 mg/ml) and control (methanol was used instead of extract) were mixed with 1.58 ml of distilled water. The test extract was assayed according to the method described above. The blank contained only methanol. All extracts were assayed in triplicate. BHA (butylated hydroxyanisole) was used as positive reference standard in the study.

3.3.2 Scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals

The scavenging activity of the extracts of *P. bleo* and *P. grandifolia* on DPPH radicals was measured according to the method of Cheung *et al*. (2003) with some modification. Extracts with different concentrations and control (methanol was used instead of extract) were mixed with 0.8 % of DPPH solution. The reaction mixtures were incubated at room temperature and allowed to react for 30 min in the dark. All measurements were done in dim light. The optical density was measured at 520 nm with a spectrophotometer. Methanol was used as blank.

The extracts were pre-screened for scavenging activity towards the stable DPPH radicals at concentrations of 1-5 mg/ml. Reaction mixtures of test extracts; DPPH and methanol for the assay were prepared according to the Table 3.2. For the IC_{50} value less than 1.0 mg/ml which was the lowest concentration in pre-screening test, the extract was subjected to further examinations on more intensive concentration (100-1000 μg/ml) by DPPH assay according to the Table 3.3.

Ascorbic acid and BHA were used as positive reference standards. The standard plot, using positive reference standards with concentrations ranging from 1.56 to 200.00 μg/ml was prepared. Reaction mixtures of positive reference standards, DPPH and methanol for the assay were prepared according to the Table 3.4.

The scavenging activity (%) on DPPH radical was calculated according to the following equation:

$$\text{Scavenging activity (\%)} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100\ \%$$

OD control was the absorbance of the control, and OD sample was the absorbance of the extract/standard. In this study, the scavenging activity was stated in the value of $IC_{50.}$ The IC_{50} value is the effective concentration of which DPPH radical was scavenged by 50 %. The IC_{50} values were estimated by a non linear regression algorithm and presented as the mean of triplicate analysis. Low IC_{50} value indicates strong ability of the extract to act as DPPH scavenger. The higher the IC_{50} value indicates the lower the scavenging activity of the scavengers as more amount of the scavengers were required to achieve 50 % scavenging reaction and thus the scavengers are less effective in scavenging the DPPH radicals.

Table 3.2: Preparation of reaction mixture of selected extracts or fractions, DPPH and methanol

Concentration of extract (mg/ml)	Volume of methanol (μl)	Volume of extract stock solution(μl)	Volume of DPPH solution (μl)
5	725	250	25.0
4	775	200	25.0
3	825	150	25.0
2	875	100	25.0
1	925	50	25.0
Control	975	-	25.0

Table 3.3: Preparation of reaction mixture of selected extracts, DPPH and methanol

Concentration of extract (μg/ml)	Volume of methanol (μl)	Volume of extract stock solution (μl)	Volume of DPPH solution (μl)
1000	925	50	25.0
900	930	45	25.0
800	935	40	25.0
700	940	35	25.0
600	945	30	25.0
500	950	25	25.0
400	955	20	25.0
300	960	15	25.0
200	965	10	25.0
100	970	5	25.0
Control	975	-	25.0

Table 3.4: Reaction mixture of positive reference standards, DPPH and methanol for DPPH assay

Concentration of positive reference standard (μg/ml)	Volume of methanol (μl)	Volume of positive reference standard stock solution (μl)	Volume of DPPH solution (μl)
200.00	475.00	500.00	25.0
100.00	725.00	250.00	25.0
50.00	850.00	125.00	25.0
25.00	912.50	62.50	25.0
12.50	943.75	31.25	25.0
6.25	959.38	15.63	25.0
3.12	967.19	7.81	25.0
1.56	971.09	3.91	25.0
Control	975.00	-	25.0

3.3.3 Reducing power assay

The reducing power of the prepared extracts was determined according to method of Oyaizu (1986). Briefly, each extract in varying amounts of 5 mg, 10 mg and 20 mg was dissolved in 1.0 ml of distilled water to which was added 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1 % (w/v) solution of potassium ferricyanide (Sigma). The mixture was incubated in a water bath at 50^0C for 20 min. Following this, 2.5 ml of 10 % (w/v) trichloroacetic acid solution was added and the mixture was then centrifuged at 1000 rpm for 10 min. A 2.5 ml aliquot of the upper layer was combined with 2.5 ml of distilled water and 0.5 ml of a 0.1 % (w/v) solution of ferric chloride. Absorbance of the reaction mixture was read spectrophotometrically at 700 nm. Increased absorbance of the reaction mixture indicates greater reducing power. Mean values from three independent samples were calculated for each extract. Ascorbic acid and BHA were used as positive reference standards. The higher absorbance of the reaction mixture indicates greater reducing power. The extracts that showed comparable absorbance readings with ascorbic acid and BHA are considered having high reducing power.

3.3.4 ß-Carotene bleaching method

The antioxidant activity of the prepared extracts was determined according to the ß-carotene bleaching method described by Cheung *et al.* (2003). A reagent mixture, containing 1 ml of ß-carotene solution (0.2 mg/ml in chloroform), 0.02 ml of linoleic acid and 0.2 ml of Tween 80 was pipette into a round-bottom flask. After removing the chloroform by using a rotary evaporator, 50 ml of oxygenated distilled water was then added to the flask. The mixture was stirred vigorously to form a liposome solution.

Aliquots (5 ml) of the liposome solution were transferred to a series of test tubes containing 0.2 ml of extract with different concentrations (4 to 20 mg/ml). Methanol or water (instead of extract) was used as control while the blank contained all the earlier chemicals (0.02 ml of linoleic acid and 0.2 ml of Tween 80 in 50 ml of oxygenated distilled water) except ß-carotene solution. The absorbance of each extract was measured immediately (t = 0 min) at 470 nm using a spectrophotometer. Subsequently, the reaction mixtures were incubated at 50 ℃. The absorbance was measured again at time intervals of 20 min for 2 h (t = 120 min). All samples were assayed in triplicate. BHA was used as positive reference standard in this assay.

The rate of ß-carotene bleaching (R) was calculated according to equation as below:

$$R = \frac{\ln (a/b)}{t}$$

where: ln = natural log; a = absorbance at time 0; b = absorbance at time *t*, which *t* = 20, 40, 60, 80, 100 or 120 min. The antioxidant activity (%) was calculated in terms of percent inhibition relative to the control, using the equation as below:

$$\text{Antioxidant activity (\%)} = \left(\frac{R_{control} - R_{sample}}{R_{control}} \right) \times 100\ \%$$

3.3.5 Statistical analysis

The antioxidant data were subjected to one-way analysis of variance (ANOVA) and the significance of the difference between the means was determined by the Duncan's multiple range tests at 95 % least significant difference ($p < 0.05$).

3.4 Antimicrobial activity

3.4.1 Preparation of agar and broth

Mueller Hinton agar

38 g of Mueller Hinton agar was suspended in 1 litre of distilled water. The solution was boiled to dissolve the medium completely and sterilized by autoclaving for 15 min at 15 psi, 121 ℃. Subsequently, the medium was cooled to 45-50 ℃ and dispensed into petri dishes. Final pH should be 7.4 ± 0.2 at 37 ℃.

Mueller Hinton broth

23 g of Mueller Hinton broth was suspended in 1 litre of distilled water. The solution was sterilized by autoclaving for 15 min at 15 psi, 121 ℃. Final pH should be 7.4 ± 0.2 at 37 ℃.

3.4.2 Test microorganisms and microbial culture

Four bacterial strains were used in this study: Gram negative bacteria included *Escherichia coli*, *Pseudomonas aeruginosa* and Gram positive bacteria included *Staphylococcus aureus*, *Bacillus subtilis*. These test microorganisms were obtained from the Microbiology Laboratory, Microbiology Division, Institute of Biological Sciences, Faculty of Science, University of Malaya, Malaysia. Bacterial strains were cultivated at 37±2 ℃ and maintained on nutrient agar (Difco, USA) slant at 4±2 ℃.

3.4.3 Agar disc diffusion assay

Preparation of test discs

The crude methanol and fractionated extracts (hexane and ethyl acetate) were dissolved in 10 % aqueous DMSO (not toxic to germs at this percentage (Berahou *et al*., 2007) and the water extracts were dissolved in distilled water to two different concentrations of 50 and 500 mg/ml. The extract solutions were then sterilized by filtration through a 0.45 µm membrane filter. The sterile discs (6 mm in diameter) were impregnated with 10 µl of the extracts (50 and 500 µg/disc). Negative controls were prepared using discs impregnated with 10 % aqueous DMSO (solvent control) and virgin discs (blank control). Standard discs of the antibiotic gentamycin (10 µg) and ampicilin (10 µg) were used as the positive antibacterial controls for all bacterial strains.

Preparation of inocula

The inocula of bacterial strains were prepared from 18 hour-old culture and suspensions were adjusted to 0.5 McFarland standard turbidity [~10^6 colony forming units (CFU) per millilitre] using McFarland Nephelometer standard according to the National Committee for Clinical Laboratory Standards (Ezoubeiri *et al*., 2005).

Procedure of agar disc diffusion assay

Antimicrobial activity was determined against four bacterial pathogens according to agar disc diffusion assay of Berahou *et al*. (2007) and National Committee for Clinical Laboratory Standards (2005), with some modifications. Petri plates were prepared by pouring 20 ml of Mueller Hinton agar. The inoculum was spread on the top of the solidified media, using sterile swab to ensure thorough

coverage of the plates and a uniform thick lawn of growth following incubation. The plates were then allowed to dry for 10 min to facilitate absorption of the broth into the agar. The discs were placed on the surface of the inoculated agar and the plates were left 30 min at room temperature to allow the diffusion of the extract before their incubation for 24 h at 37±2 ℃.

The antibacterial activity was evaluated by measuring the zone of complete growth inhibition surrounding the discs (Figure 3.1). The diameters of inhibition zones were measured in millimetres using Vernier Calipers. The diameter of inhibition zones reported here including the diameter of the disc (6 mm). All tests were repeated thrice to minimize test error. An inhibition zone of 14 mm or greater (including diameter of the disc) was considered as high antibacterial activity (Ramzi and Ulrike, 2005).

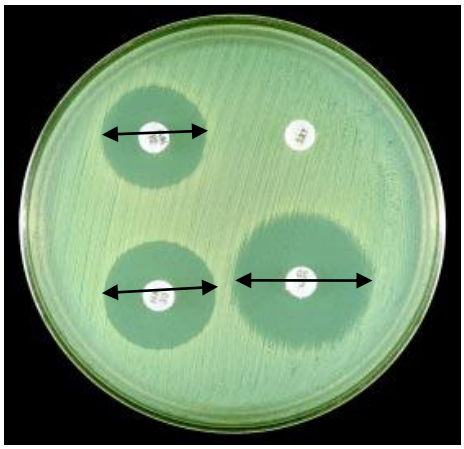


Figure 3.1: Example of the agar disc diffusion assay. The diameters of inhibition zones (including the diameter of the disc) were measured in millimetres.

3.4.4 Broth dilution assay

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts were determined by broth dilution assay according to the method of Furiga *et al*. (2009) with some modifications.

Determination of minimum inhibitory concentration (MIC)

Briefly, bacterial strains were cultured overnight on Mueller Hinton broth and adjusted to a final density of 0.5 McFarland standard turbidity (~10^6 colony forming units (CFU) per millilitre) using McFarland Nephelometer standard according to the National Committee for Clinical Laboratory Standards. Then, the cultures were inoculated into tubes containing extract dilutions to obtain the following final concentration: 10, 20, 30, 40 and 50 mg/ml for each tested extracts and incubated at 37 ℃ for 24 h. Blank control was prepared by virgin media. Each MIC experiment was repeated three times. Inhibition of bacterial growth in the test tubes containing tested extracts was judged by comparison with growth in blank control plates. The MIC was defined as the lowest concentration which no visible growth was observed. Extracts with MIC values < 10 mg/ml were considered active.

Determination of minimum bactericidal concentration (MBC)

For MBC determination, an aliquot (10 µl) from each tubes of tested extracts and controls in MIC determination was cultured onto Mueller Hinton agar plates after incubation to determine if the inhibition was reversible or permanent. The plates were

then incubated at 37 ℃ for 24 h. Each MIC experiment was repeated three times. Blank control was prepared by virgin media. The MBC was defined as the lowest concentration of tested extracts that did not permit any visible growth after the incubation period.

3.4.5 Statistical analysis

All samples were prepared in triplicate for comparison of values. All data were recorded as means ± standard deviation. Statistical analysis was carried out with Microsoft excel.

3.5. *In vitro* cytotoxicity assay

3.5.1 Cell lines and culture medium

Human nasopharyngeal epidermoid carcinoma cell line (KB), human cervical carcinoma cell line (CasKi), human colon carcinoma cell line (HCT 116), hormone-dependent breast carcinoma cell line (MCF7), human lung carcinoma cell line (A549) and non-cancer human fibroblast cell line (MRC-5) were purchased from the American Tissue Culture Collection (ATCC, USA). The viability of the cells was checked before and after treatment by the trypan blue exclusion dye method. Frozen cell stocks were stored in liquid nitrogen (-196 ℃) prior to use.

KB cells were maintained in Medium 199 (Sigma); CasKi, A549 and MCF7 cells in RPMI 1640 medium (Sigma); HCT 116 in McCOY'S 5A Medium (Sigma) and MRC-5 cells in EMEM (Eagle Minimum Essential Medium) (Sigma), supplemented

with 10% foetal bovine serum (FBS, PAA Lab, Austria), 100 µg/ml penicillin or streptomycin (PAA Lab, Austria) and 50 µg/ml of fungizone (PAA Lab, Austria) (Appendix A, no.1). The cells were cultured in a 5 % CO_2 incubator (Shel Lab water-jacketed) kept at 37 ℃ in a humidified atmosphere. The culture was subcultured every two to three days as needed and routinely checked under an inverted microscope (IMT-2 Olympus, Japan) for any contamination (Appendix A, no.4).

3.5.2 *In vitro* neutral red cytotoxicity assay

The neutral red cytotoxicity assay is based on the initial protocol described by Borenfreund and Puerner (1984) with some modifications and it determines the accumulation of the neutral red dye in the lysosomes of viable and uninjured cells.

Briefly, cells were detached from the tissue culture flask with 0.25 % trypsin-EDTA solution and PBS solution and washed in culture medium. The cell pellet was obtained by centrifugation at 1000 rpm for 5 min. The density of the viable cells was counted by 0.4 % of tryphan blue (Appendix A, no.2) exclusion in a haemocytometer. The cells ($1x10^4$/well) were then plated in a 96-well microtiter plate (Nunc) in a volume of 190 µl. The plate was incubated in a CO_2 incubator at 37 ℃ for 24 h to allow the cells to adhere and achieve 60-70 % confluence at the time of the addition of the test agents (i.e. test extracts and test compounds). After 24 h of incubation, the cells were treated with different concentrations (1, 10, 50 and 100 µg/ml) of each test agents in three replicate tests. The plates were incubated for 72 h at 37 ℃ in a 5 % CO_2 incubator. DMSO was used to dilute the test agents and the final concentration of DMSO in test wells and control wells used was not in excess of 1 % (v/v). No effect due to the

DMSO was observed. Doxorubicin was used as the positive control. The well containing untreated cells is the negative control.

At the end of the incubation period, the media were replaced with medium containing 50 µg/ml neutral red. The plates were incubated for another 3 h to allow for uptake of the vital dye into the lysosomes of viable and uninjured cells. After the incubation period, the media were removed and cells were washed with the neutral red washing solution. The dye was eluted from the cells by adding 200 µl of neutral red resorb solution and further incubated for 30 min at room temperature with rapid agitation on a microtiter plate shaker (LT BioMax 500). Dye absorbance was measured at 540 nm using ELISA reader (Titertek Multiskan MCC/340). Three replicate plates were used to determine the cytotoxicity activity of each test agent. The cytotoxic effect of each test agent was evaluated based on percentage inhibition values.

The percentage of inhibition (%) of was calculated according to the following formula:

$$\text{Percentage of inhibition (\%)} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100\%$$

The average of three replicates was then obtained. Cytotoxicity of each test agent is expressed as IC_{50} value. The IC_{50} value is the concentration of test agents that cause 50 % inhibition or cell death, averaged from the three experiments, and was obtained by plotting the percentage inhibition versus concentration of test agents. According to US NCI (United States National Cancer Institute) plant screening program, a plant extract is generally considered to have active cytotoxic effect if the IC_{50} value, following incubation between 48 to 72 h, is 20 µg/ml or less, while it is 4 µg/ml or less for pure compounds (Lee and Houghton, 2005; Geran *et al*., 1972).

3.5.3 Statistical analysis

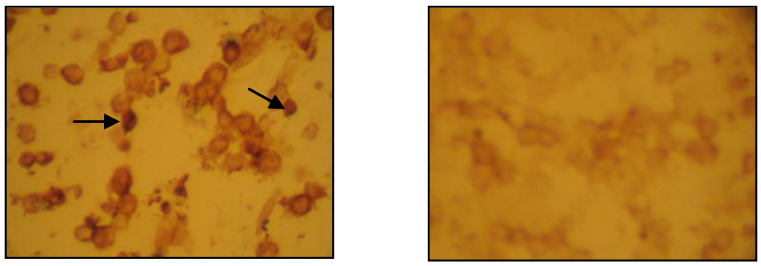
All samples were prepared in triplicate for comparison of values. All data were recorded as means ± standard deviation. Statistical analysis was carried out with Microsoft excel.

3.6 Detection of DNA fragmentation (apoptosis)

Cell culture and maintenance of cell were described in Appendix A (no.4). DNA fragmentation was detected using DeadEnd^{TM} Colorimetric Apoptosis Detection System as described by the manufacturer (Promega). Briefly, cells ($5x10^5$ cells/ml) were subcultured into 24 well plates and incubated for 12-24 h to allow the cells to adhere. When the cells reached confluency between 60-70 %, the medium was replaced with fresh medium. Subsequently, the cells were stimulated with the tested extract or compound at concentration required for 50 % inhibition growth of cancer cells (refer to section 4.4.3 and 4.9) for 24 h. Only extracts or compounds which were active in cytotoxic activity were further evaluated for detection of DNA fragmentation. Control cells were treated with the same final concentration of DMSO present in the treated wells. Positive control cells were treated with DNase I.

After stimulation, the cells were fixed with 4 % (w/v) paraformaldehyde in PBS (pH 7.4) for 25 min at room temperature and rinsed twice with PBS. Then, the cells were immersed in 0.2 % (v/v) Triton X-100 solution for 5 min and rinsed with PBS. The cells were then incubated in 100 µl equilibration buffer (supplied with the kit) for 5–10 min. Subsequently, 100 µl of reaction buffer containing TdT enzyme and biotinylated nucleotide mix was added to the cells, covered with coverslips and

incubated for 1 h at 37 ℃. The reaction was terminated with by immersing the slide in 2× SSC for 15 min followed by washing with PBS and 0.3 % (v/v) hydrogen peroxide for 5 min. The slides were then incubated with 100 µl sterptavidin HRP solution for 30 min, rinsed with PBS and finally incubated with DAB components until a light brown background developed. The stained cells were immediately observed under the light microscope. Using this procedure, apoptotic nuclei were stained dark brown. Example of the induction of apoptosis by the DNase I (positive control) and DMSO (negative control) are showed in Figure 3.2.



(A) **(B)**

Figure 3.2: Induction of apoptosis by the DNase I and DMSO on the KB cells. KB cells were treated with DNase I (positive control, A) and DMSO (negative control, B) for 24 hrs and subjected to DeadEnd™ Colorimetric Apoptosis Detection System (Promega). Dark stained nuclei (arrows) of the KB cells were observed after cell treatment with DNase I whereas no stained nucleus in cells treated with DMSO was detected. A-B: 400x magnification.

3.7 Determination of the expression level of apoptotic-related genes

The mRNA expression levels of widely established apoptotic-related genes, i.e. p53, caspase-3 and c-myc were carried out by real-time reverse transcriptase-polymerase chain reaction (RT-qPCR) using LUX (Light Upon eXtension) primers. The assay was started with preparation of quantitative standard (QSTD) (section 3.7.1), followed by validation and optimization of qPCR assay using LUX primers (section 3.7.2) and application of optimized qPCR assay to experiment set (section 3.7.3).

3.7.1 Preparation of quantitative standard (QSTD) for real-time quantification

The quantitative standards (QSTD) of widely established apoptotic-related genes (p53, caspase-3, c-myc) and ß-actin were prepared by using one-step RT-PCR. These QSTD were used as DNA templates for optimization of RT-qPCR and also to prepare standard curve for the RT-qPCR assay after further diluted in 10-fold with diethyl pyrocarbonate (DEPC) treated water. The inclusion of standard curve on every run is an important control to measurement.

Cell culture

Cell culture and maintenance of cell were described in Appendix A (no.4). Briefly, untreated cells ($1x10^6$ cells/well) were sub-cultured into 24 well plates and incubated for 4 h before RNA extraction.

RNA extraction

For QSTD preparation, total cellular RNA was isolated from the untreated cells using RNeasy minikit (Qiagen) according to manufacturer's protocol. Briefly, the cells were trypsinized and collected as a cell pellet in a 1.5 ml microcentrifuge tube prior to lysis. The cell pellet was loosened thoroughly by flicking the tube. 350 µl of Buffer RLT was added to the cell pellet and mixed by pulse-vortexing for 15 seconds. RNA was precipitated by adding 1 volume (350 µl) of 70 % ethanol (molecular grade) to cell lysate and mixed well gently by pipetting. 700 µl of the solution was then transferred to an RNeasy spin column placed in a 2 ml collection tube (supplied) and centrifuge at 10000 rpm for 15 seconds (the flow-through was discarded after centrifuge).

Subsequently, 700 µl of Buffer RW1 was added to the RNeasy spin column and centrifuge at 10000 rpm for 15 seconds to wash the spin column membrane (the flow-through was discarded after centrifuge). Then, 500 µl of Buffer RPE was added to the RNeasy spin column and centrifuge at 10000 rpm for 15 seconds (the flow-through was discarded after centrifuge). This step was repeated with the longer centrifuge time (2 min). This long centrifugation dried the spin column membrane, ensuring that no ethanol was carried over during RNA elution as residual ethanol might interfered with downstream reactions. After centrifugation, the RNeasy spin column was carefully removed from the collection tube so that the column did not contact with the flow through to prevent the carryover of ethanol. The RNeasy spin column was placed in a new 2 ml collection tube and centrifuge at full speed (~14 000 rpm) for 1 min. This is to eliminate any possible carryover of Buffer RPE. Finally, the RNeasy spin column was placed in a new 1.5 ml collection tube (DNase/RNase free) and 50 µl of RNase free water was added directly to the spin column membrane. The spin column and the collection tube were then centrifuge at 10000 rpm for 1 min to elute the RNA. The eluted RNA was then stored in -70 ℃ till used.

Determination of nucleic acid concentrations

Nucleic acid quality encompasses both its purity (absence of protein and DNA contamination, absence of inhibitors) and its integrity. The concentration of extracted nucleic acid was determined by measuring the absorbance using a spectrophotometer (Eppendorf BioPhotometer plus). The quantity of the nucleic acid was determined at a wavelength of 260 nm.

The quality of RNA is very important for the reproducibility and biological relevance of the subsequent quantitative real-time PCR (qPCR). The ratio between the

absorbance values at 260 and 280 nm (A_{260}/A_{280}) provided an estimate of the purity of RNA with respect to contaminants that absorb in the spectrum, such as protein. The pure RNA has an A_{260}/A_{280} ratio of 1.9-2.1.

One step RT-PCR

The extracted RNA was reversed transcribed into cDNA and used as the template for PCR amplification with OneStep RT-PCR kit (Qiagen). Briefly, the RT-PCR was carried out in a final volume of 20 µl containing 1x PCR Buffer (with $MgCl_2$), 400 µM of each dNTP, 0.25 µM of each primer, OneStep RT-PCR enzyme mix, 1 µg of RNA template and appropriate volume of RNase-free water. The preparation of master mix for RT-PCR is shown in Table 3.5.

Table 3.5: Reaction components for one-step RT-PCR

Component	Volume/reaction (µl)	Final concentration
Master mix 5x QIAGEN OneStep RT-PCR Buffer*	4	1x
dNTP Mix (containing 10 mM of each dNTP)	0.8	400 µM of each dNTP
Primer mix (10 µM)**	0.5	0.25 µM of each primer
QIAGEN OneStep RT-PCR Enzyme Mix	1	-
RNase-free water	11.7	-
Template RNA	5	Approximately 1 µg/reaction
Total volume	20	-

*Contains 12.5 mM $MgCl_2$

**Primer mix was prepared by mixing each forward and reverse primer with appropriate volume of DEPC treated distilled water and dissolving the lyophilized pellet in tube by vortexing followed by a brief spin.

The thermal cycling profile of RT-PCR assay consisted of 30 min reverse transcription (RT) step performed at 50 ℃, 15 min of initial PCR activation step (*Taq* polymerase activation) at 95 ℃, followed by 40 cycles of PCR at 95 ℃ denaturation for 30 seconds, 50 ℃ of annealing for 40 seconds and 72 ℃ extension for 1 min, and terminated with an additional extension step for 5 min at 72 ℃. The RT-PCR was performed in LabCycler 96-well Gradient Thermalcycler (SensoQuest). The thermal cycling profile is outlined in Table 3.6.

Table 3.6: Thermal cycling profile of one-step RT-PCR

Step	
Reverse transcription	50 ^{o}C 30 min
Initial PCR activation step	95 ^{o}C 15 min
3step cycling:	
Denaturation	95 ^{o}C 30 s
Annealing	50 ^{o}C 40 s
Extension	72 ^{o}C 1 min
Number of cycle	40
Final extension	72 ^{o}C 5 min
Indefinite	4 ^{o}C

The oligonucleotide sequences of the PCR primers used herein were designed based on the human mRNA encoding the respective genes (Table 3.7). The PCR products were analyzed by gel electrophoresis. 5 µl aliquot of the 20 µl PCR product was loaded to a 1.25 % (w/v) agarose gel (Sea Kem LE agarose) in 1X TBE buffer with a 100-bp ladder as molecular weight marker. The results were scored as positive or negative by the presence or absence of a band.

Table 3.7: The sequence of primers used in RT-PCR for preparation of QSTD

Primer	Strand	Sequence	GC %	Tm	Reference
p53	Forward	TGT GGA GTA TTT GGA TGA CA	40	50.3	Tan *et al*., 2005
p53	Reverse	GAA CAT GAG TTT TTT ATG GC	35	47.5	
Caspase-3	Forward	TCA CAG CAA AAG GAG CAG TTT	42.9	54.9	Tan *et al*., 2005
Caspase-3	Reverse	CGT CAA AGG AAA AGG ACT CAA	42.9	52.7	
c-myc	Forward	GAA CAA GAA GAT GAG GAA GA	40	48.7	Tan *et al*., 2005
c-myc	Reverse	AGT TTG TGT TTC AAC TGT TC	35	49.0	
ß-Actin	Forward	TCA CCC TGA AGT ACC CCA TC	55	54.7	Tan *et al*., 2005
ß-Actin	Reverse	CCA TCT CTT GCT GCA AGT CC	55	55.4	

Specificity test of primer

Before further proceed to PCR product purification, specificity of the primers need to be confirmed. The PCR products were checked for undesired or unspecific sequences. Primer specificity was evaluated by performing PCR with single set of primers against the QSTD for each gene (i.e. p53, caspase-3, c-myc and ß-actin). The PCR was carried out as the 3 step cycling profile described in Table 3.6. The PCR products were analyzed by gel electrophoresis. 5 µl aliquot of the 20 µl PCR product was loaded to a 1.25 % (w/v) agarose gel (Sea Kem LE agarose) in 1X TBE buffer with a 100-bp ladder as molecular weight marker. The results were scored as positive or negative by the presence or absence of a band.

PCR product purification

The PCR product was purified using simple ethanol precipitation method. Firstly, 1 ml of cold absolute ethanol (molecular grade) was added to the PCR product and mixed well gently by pipetting. Subsequently, the tube contained the solution was centrifuge at full speed (approximately 14 000 rpm) for 15 min and a pellet was precipitated at the bottom of the tube after centrifuge. The flow-through was discarded gently after centrifuge. The pellet was then washed twice with 70 % ethanol (molecular grade). Lastly, the pellet was air-dried and dissolved in 50 µl RNase-free water.

Actually, there are many alternative protocols (e.g., Qiagen columns, Microcon filters). However, this is the cheapest and most reliable protocol.

Quantification of DNA

The concentration of purified PCR product was determined by measuring the absorbance using a spectrophotometer (Eppendorf BioPhotometer plus). The ratio between the absorbance values at 260 and 280 nm (A_{260}/A_{280}) provided an estimation of the purity of DNA with respect to contaminants that absorb in the spectrum, such as protein. The pure DNA has an A_{260}/A_{280} ratio of 1.8. The QSTD were stored in -70 ℃ till used.

From the reading of spectrophotometer (µg/ml or ng/µl), the copy numbers of the QSTD for each genes (i.e. p53, caspase-3, c-myc and ß-actin) were calculated using the following equation:

$$\text{Number of copies/µl} = \frac{\text{DNA concentration (g/µl)} \times 6.023 \times 10^{23}}{\text{PCR product length (bp)} \times 640}$$

The 6.023×10^{23} is the number of copies per mole while 640 is a constant for dsDNA.

These copy numbers were important for preparation of standard curve in qPCR assay. Series of 10-fold dilutions of QSTD were later being prepared using DEPC treated water and the template was ranging from 10^3 to 10^{10}. These dilutions were amplified with qPCR according to the procedure stated in '*Amplification of qPCR*' under section 3.7.2. Linear regression equations for obtained Ct values were calculated.

3.7.2 Validation and optimization of qPCR assay using LUX primers

The PCR method used for preparation of QSTD was adapted to real-time format by using LUX primers (Invitrogen), Platinum *Taq* DNA polymerase (Invitrogen) and dNTP mix (Promega). The qPCR assays were performed in Rotor-Gene 6000

(Corbette). For the optimization of the qPCR assay, QSTD prepared in section 3.7.1 was used as DNA template.

LUX primers

The sequences of the fluorogenic forward primer and the corresponding unlabeled reverse primer were designed and ordered using proprietary software called D-LUX Designer (Invitrogen, http://www.invitrogen.com/lux). The characteristics of the LUX primers, such as length and Tm, are included in the primer designed by the software to output primer pairs that are located throughout the target (input) sequence. The sequences of the primers were checked using the NCBI Blast software.

Each fluorogenic LUX primer is labelled with one of two reporter dyes, either FAM (6-carboxy-fluorescein) or JOE (6-carboxy-4',5'-dichloro-2',7'-dimethoxy-flurescein). In the present study, FAM-labelled primer set was used to detect genes of interest (p53, caspase-3 and c-myc) while a JOE-labelled primer set was used to detect a housekeeping gene (ß-actin) as an internal control. The sequence of LUX primers used in the present qRT-PCR study is shown in Table 3.8.

Table 3.8: The sequence of LUX primers used in qPCR

Primer	Strand	Sequence	Dye label	GC %	Tm	Amplicon length
p53	Reverse	cgagcGTGTTGTTGGGCAGTGCTcG	FAM	60	67.55	84
p53	Forward	AAGAGAATCTCCGCAAGAAAGG		45.45	61.18	
Caspase-3	Forward	cggcTAAAATACCAGTGGAGGCcG	FAM	50	62.12	84
Caspase-3	Reverse	CATCCTTTGAATTTCGCCAAGA		40.91	63.44	
c-myc	Forward	cgccGAGGAGAATGTCAAGAGGcG	FAM	55	62.29	96
c-myc	Reverse	ATCTGGTCACGCAGGGCAAA		55	66.23	
ß-Actin	Reverse	cgtaccCATCACGATGCCAGTGGTAcG	JOE	57.14	67.07	68
ß-Actin	Forward	ACGTTGCTATCCAGGCTGTGC		57.14	64.78	

Amplification by qPCR

Briefly, the qPCR assay was carried out in a final volume of 10 µl. A master reagent mix that contained all the components required for qPCR except the template DNA was prepared according to the number of reactions desired (included samples, non-reverse-transcriptase control (NRTC) and non-template control (NTC). The master mix was then divided into portions of 8 µl in a 0.1 ml strip tube (Corbette). Lastly, 2 µl of nucleic acid (DNA) template was added into each tube. The preparation of master mix for qPCR is shown in Table 3.9 while the thermal cycling profile is outlined in Table 3.10.

Table 3.9: Reaction components for qPCR

Component	Volume / reaction (µl)	Final concentration
Master mix		
10x PCR Buffer*	1	1x
dNTP Mix (containing 10 mM of each dNTP)	0.5	500 µM of each dNTP
LUX primer mix**	1.5	0.3 µM
Platinum *Taq* DNA polymerase	0.15	-
$MgCl_2$ (20 mM)	1.5	3.0 mM
RNase-free water	3.35	-
Template DNA	2	-
Total volume	10	-

* Without $MgCl_2$

**LUX primer mix was prepared by mixing forward and reverse primer with appropriate volume of DEPC treated distilled water and dissolving the lyophilized pellet in tube by vortexing followed by a brief spin.

Table 3.10: Thermal cycling and melting profile of qPCR

3step cycling:		
Denaturation	95 ^{o}C	15 s
Annealing	55 ^{o}C	15 s
Extension	72 ^{o}C	20 s
Number of cycle	35	
Melting condition:		
Ramp from 72 ^{o}C to 95 ^{o}C		
Rising by 1 ^{o}C each step		
Wait for 90 s of pre-melt conditioning on first step		
Wait for 5 s for each step afterwards		

Melting curve analysis

Melting curve analysis was performed after the thermo cycling of qPCR amplification to identify the presence of primer dimmers and analyze the specificity of the reaction. In the present study, melting curve analysis was programmed to hold 90 seconds of pre-melt conditioning on first step, ramp from 72 ^{o}C to 95 ^{o}C and rising by 1 ^{o}C each step. All amplicon for a particular primer pair should have the same Tm (melting temperature), unless there are contamination, mispriming or primer dimmers artefacts. The melting profile of qPCR is outlined in Table 3.10.

Controls

Two types of negative controls were used in the present study, which were: i) non-reverse-transcriptase control (NRTC), which is a minus reverse-transcriptase control (or "No Amplification Control") that containing the master mix reagents and the

RNA template without reverse transcriptase procedure. ii) non-template control (NTC), which NTC includes all of the RT-PCR reagents except the DNA/RNA template. Both NRTC and NTC were used during the entire assay development and assay evaluation.

Validation and optimization of the LUX RT-qPCR assay

(i) Specificity test of primer

The specificity of each LUX primers (p53, caspase-3, c-myc and ß-actin) was assessed against the purified DNA of QSTD of each gene (p53, caspase-3, c-myc and ß-actin), to confirm that each set of primers amplified only the specific genome. The DNA-primer mix preparation is shown in Table 3.11, the preparation of master mix for primer specificity test is shown in Table 3.12 while the thermal cycling profile is outlined in Table 3.10.

Table 3.11: DNA-primer mix preparation for optimization assay

QSTD / Primer	c-myc	p53	caspase-3	ß-actin
c-myc	A	B	C	D
p53	E	F	G	H
caspase-3	I	J	K	L
ß-actin	M	N	O	P

Table 3.12: Reaction components for qPCR

Component	Volume / reaction (µl)	Final concentration
Master mix		
10x PCR Buffer*	1	1x
dNTP Mix (containing 10 mM of each dNTP)	0.5	500 µM of each dNTP
Platinum *Taq* DNA polymerase	0.15	-
$MgCl_2$ (20 mM)	1.5	3.0 mM
RNase-free water	3.35	-
Template DNA-LUX primer mix (A-P in Table 3.11)**	3.5	-
Total volume	10	-

*Without $MgCl_2$

**LUX primer mix was prepared by mixing forward and reverse primer with appropriate volume of DEPC treated distilled water and dissolving the lyophilized pellet in tube by vortexing followed by a brief spin.

(ii) Optimization of the concentrations of LUX primers and $MgCl_2$

The optimization were performed by using a matrix of concentrations of each LUX primer mix and concentrations of the $MgCl_2$ (Table 3.13) to determine the optimal concentrations of LUX primer and $MgCl_2$ yielding the lowest Ct values, hence, the highest amplification efficiencies. The matrix of primer-$MgCl_2$ mix is shown in the Table 3.13, the preparation of master mix for primer specificity test is shown in Table 3.14 while the thermal cycling profile is outlined in Table 3.10.

Table 3.13: Concentrations of Primer-$MgCl_2$ mix for optimization assay

[$MgCl_2$] (mM) / [Primer] (uM)	6.0	4.5	3.0	1.5
0.3	A	B	C	D
0.5	E	F	G	H
0.6	I	J	K	L

Table 3.14: Reaction components for qPCR

Component	Volume / reaction (µl)	Final concentration
Master mix		
10x PCR Buffer*	1	1x
dNTP Mix (containing 10 mM of each dNTP)	0.5	500 µM of each dNTP
Platinum *Taq* DNA polymerase	0.15	-
RNase-free water	3.35	-
$MgCl_2$-LUX primer mix (A-L in Table 3.13)**	3	-
Template DNA	**2**	
Total volume	**10**	-

*Without $MgCl_2$

**LUX primer mix was prepared by mixing forward and reverse primer with appropriate volume of DEPC treated distilled water and dissolving the lyophilized pellet in tube by vortexing followed by a brief spin.

(iii) Optimization of the annealing temperature (T_A)

The optimization of T_A was carried out as described in '*Amplification by qPCR*' under section 3.7.2, except the T_A in the thermal cycling profile. Three different T_A were tested, which were 50 ℃, 55 ℃ and 60 ℃.

Determination of the lowest detection limit and sensitivity of the LUX RT-qPCR assay

The detection limit and sensitivity of the LUX qPCR assay was assessed by determining the Ct values of serial 10-fold dilutions of purified QSTD, covering the range between 10^3 and 10^{10} copies. Standard curves prepared with these dilutions were used in every experiment of each gene.

3.7.3 Application of optimized qPCR assay to experiment set

The qPCR assay which has been optimized (section 3.7.2) was applied to the experiment set in the present study. The mRNA expression levels of the apoptotic-related genes, i.e. p53, caspase-3 and c-myc in extract-treated cells were carried out using the two-step RT-qPCR. The mRNA level of ß-actin was used as internal control for template levels. Only extracts or compounds which were active in cytotoxic activity and detected with DNA fragmentation were tested in the gene expression study.

This present two-step RT-qPCR assay used RNA which was isolated from the extract-treated cells using RNeasy minikit (Qiagen) at 6 time points as starting material in a reverse transcription reaction to generate first-strand cDNA with Sensiscript

Reverse Transcriptase kit (Qiagen). The cDNA was then quantified in separate qPCR amplification.

Cell culture

Cell culture and maintenance of cell were described in Appendix A (no.4). Briefly, cells ($1x10^6$ cells/well) were sub-cultured into 24 well plates and incubated for 4 h before stimulation. Concentration of tested extracts needed to achieve 50 % growth inhibition was used to stimulate the cells over the period of 6 h at 6 time points, which were 0 h (control), 15 min, 30 min, 1 h, 3 h and 6 h. Only extracts or compounds which were active in cytotoxic activity and detected with DNA fragmentation were tested in the gene expression study.

RNA extraction

Total cellular RNA from the treated cells was extracted using RNeasy minikit (Qiagen) according to manufacturer's protocol at 6 time points, which were 0 h (control), 15 min, 30 min, 1 h, 3 h and 6 h. The extracted RNAs were then quantified using a spectrophotometer (Eppendorf BioPhotometer plus) as mentioned in '*Quantification of DNA*' under section 3.7.1.

cDNA synthesis

To achieve comparable reaction efficiency for the reverse transcription reaction and therefore to allow the direct comparison of mRNA concentrations from different

time points of the samples, the RNA templates were standardized to contain the same amount of total RNA before reverse transcription. 50 ng of RNA template was reversed transcript into cDNA using Sensiscript Reverse Transcriptase kit (Qiagen) according to manufacturer's protocol. Briefly, PCR was carried out in a final volume of 20 µl containing 1x Buffer RT, 0.5 mM of each dNTP, 1 µM of Oligo-dT primer, Sensiscript reverse transcriptase and appropriate volume of RNase-free water. The preparation of master mix for RT-PCR is shown in Table 3.15.

Table 3.15: Reaction components for cDNA synthesis

Component	Volume / reaction (µl)	Final concentration
Master mix		
10x Buffer RT	2	1x
dNTP Mix (containing 5 mM of each dNTP)	2	0.5 mM of each dNTP
Oligo-dT primer (10 µM)*	2	1 µM
Sensiscript reverse transcriptase	1	-
RNase-free water	11	-
Template RNA	2	50 ng/reaction
Total volume	20	-

*the Oligo-dT primer (Qiagen) was not provided in kit.

The cDNA synthesis step was performed in LabCycler 96-well Gradient Thermalcycler (SensoQuest). The synthesis profile consisted of 60 min incubation at 37 ℃ and inactivation of Sensiscript reverse transcriptase at 93 ℃ for 5 min, followed by rapid cooling on ice.

qPCR amplification

The PCR amplification was performed in a total volume of 10 µl as described in section 3.7.2. Briefly, the master reagent mix that contained all the components required for qPCR except the template DNA was prepared according to the number of reactions desired (included samples, standards, non-reverse-transcriptase control [NRTC] and non-template control [NTC]). The master mix was then divided into portions of 8 µl in a 0.1 ml strip tube (Corbette). Lastly, 2 µl of nucleic acid (DNA) template was added into each tube. The preparation of master mix for qPCR is shown in Table 3.9. The series of 10-fold dilutions of QSTD were used to prepare the standard curve.

Thus, every qPCR assay of each extract (or compound tested) for each gene needed 42 reactions for every run, which included 6 unknowns (cDNAs of samples at 6 time points), 8 QSTD template control (to prepare standard curve), 6 NRTC (non-RT controls for the samples at 6 time points) and 1 NTC. All reactions were performed in duplicates.

Lastly, the data was analyzed with Rotor-Gene 6000 (Corbette) software. The results of samples were projected on the standard curve generated with series dilution of QSTD.

3.7.4 Statistical analysis

In the present study, relative quantitation using two standard curves method (or relative fold change method) was used to quantitate alterations in mRNA levels using RT-qPCR. The ß-actin used as internal control was compared to a gene of interest (p53, caspase-3 or c-myc) for every sample at 6 time points. A sample (at every time point)

therefore has to be analyzed twice, firstly with the gene of interest and secondly with the internal control. Two standard curves have to be run to obtain two values (internal control and gene of interest) for the one sample.

For each standard curve, the relative standard consisted samples and calibrator (which is the QSTD in this study), which was used to create a dilution series with arbitrary units. Each standard curve was generated by performing 8 independent serial dilutions of the QSTD template, together with samples (at different time points) and negative control reactions (NTC and NRTC). Each concentration of QSTD, samples and negative controls for standard curve preparation was assaying in duplicate to allow a determination of reproducibility. To maximize accuracy, the dilutions of the QSTD should be made over the range of copy numbers that include the amount of target mRNA expected in the experimental RNA samples (example: Figure 3.3).

The copy numbers of the QSTD were entered in the setting; the dilution curve, NTC, NRTC and unknowns (experimental samples were entered into the setting as unknown) that needed to be quantitated using the standard curve generated with QSTD were also defined in the setting. The copy numbers of experimental samples were calculated after real-time amplification from the linear regression of that standard curve. The real-time software (Rotor-Gene 6000 series software) was then calculated PCR efficiency value for every standard curve, analyzed the data and reported the copy number of DNA in each of the unknown samples (example: Figure 3.3).

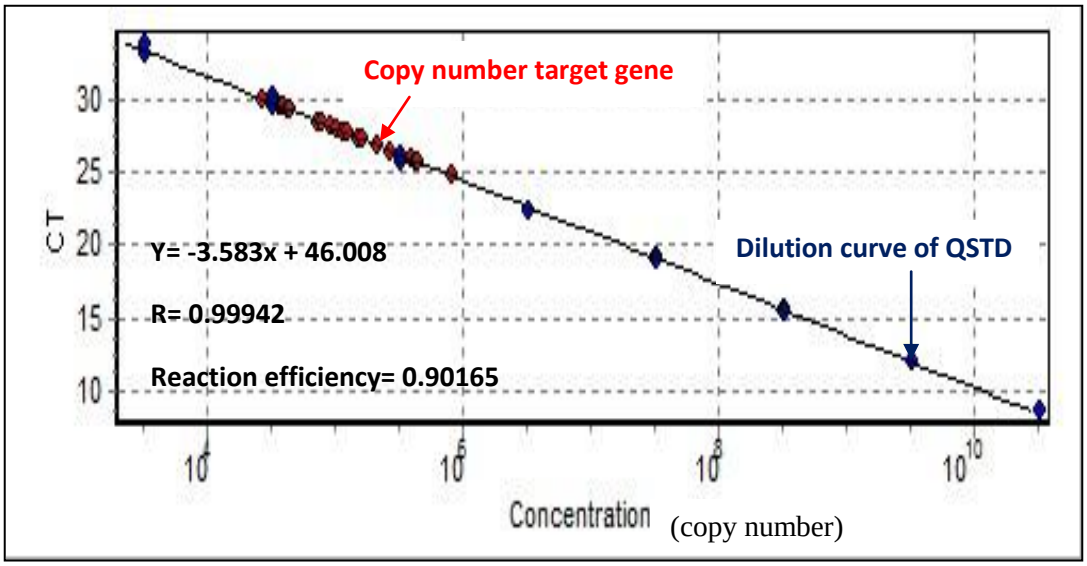


Figure 3.3: Result analyzed by the software

Lastly, the data of copy numbers obtained from the two standard curves were then exported to Excel using the export function in the software. The copy number of the experimental gene (p53, caspase-3 and c-myc) at each point of time was normalized relative to that from ß-actin and the ratio in untreated sample was assigned as 1.

The results were expressed as the means ± standard deviation. Statistical significance of the difference among treatments was determined using the Student's *t*-test. The *p*-value < 0.05 was considered statistically significant.

3.8 Acute oral toxicity

3.8.1 Test animals

Experiment was performed on healthy ICR (Imprinting Control Region) mice (5 weeks of age, body weight 23-28 g), obtained from Laboratory Animal Centre, Faculty of Medicine, University of Malaya. The female mice were confirmed nulliparous and

non-pregnant. The mice were assigned to five dosage groups and one control groups with ten mice (five male and five female) for each test group. The weight variation of mice used on study did not exceed ± 20 % SD of the mean body weight of each sex. The experimental procedures involving animals were approved by the University of Malaya Animal Experimental Ethnics Committee [Ethical number: ISB/05/08/2009/SKS (R)].

3.8.2 Procedure of acute oral toxicity

The acute oral toxicity of the crude methanol extracts of both *Pereskia* species were evaluated in mice using the procedure described by OECD (Organization for Economic Co-operation and Development, 1998) with some modifications. The mice were housed in suspended, stainless steel, wire-mesh cages in experimental animal room. The temperature was maintained at 23 ± 3 ℃ and relative humidity was 50-60 % before and after treatment with extract. The animal room was artificially illuminated (fluorescent light) on an approximate 12-hr light/dark cycle. The mice were acclimatized to the laboratory conditions for at least five days prior to commencement of the experiments. The mice were randomly selected for use in the study and marked to provide individual identification. Conventional mouse diets with unlimited supply of drinking water were available *ad libitum* except during the fasting period. The mice were starved approximately 12 hrs prior to dosing, but they had free access to drinking water. Before and after treatment with extract, the mice were caged in groups by sex and dose levels. The extracts were suspended in vehicle (10 % Tween-80 in distilled water). Stock concentration of 200 mg/ml was prepared and the mice were administrated with 0.2 ml of extract for every 10 g of the mice body weight. The mice

were administered with doses of 500, 1000, 1500, 2000 and 2500 mg/kg of extracts. Food was returned to the animals approximately 3-4 hrs after dosing. The mice were observed carefully for any signs of toxicity for the first 4 hours after the treatment period, and daily thereafter for a period of 14 days. Observations for mortality, signs of illness, injury, pain, distress, allergic reactions, changes of outer appearance, behavioural alterations (i.e. ataxia, hyperactivity, hypoactivity) and general stimulation or sedation were conducted twice daily. The observations were recorded systematically; individual records were maintained for each mouse.

3.9 Extraction, isolation and identification of chemical constituents from the bioactive extracts

3.9.1 Instrumentation

NMR (Nuclear Magnetic Resonance) spectra were recorded on a JOEL 400 MHz FT NMR spectrometer at 400 MHz for ^{1}H NMR (^{1}H Nuclear Magnetic Resonance) and at 100.40 MHz for ^{13}C NMR (^{13}C Nuclear Magnetic Resonance). Internal standards used in ^{1}H NMR spectra was TMS (δ: 0.00) for $CDCl_3$; in ^{13}C NMR was $CDCl_3$ (δ: 77.0).

GCMS (Gas Chromatography and Mass Spectra) analysis was performed using Agilent Technologies 6980N gas chromatograph equipped with a 5979 Mass Selective Detector (70 eV direct inlet); a HP-5ms (5 % phenyl methyl siloxane) capillary column (30.0 m x 250 μm x 0.25 μm) initially set at 60 °C for 10 min, then programmed to 230 °C at 3 °C min^{-1} and held for 1 min at 230 °C using helium as the carrier gas at a flow rate of 1 ml min^{-1}.

The total ion chromatogram obtained was auto integrated by ChemStation and the components were identified by comparison with an accompanying mass spectral database (NIST 05 MS Library, 2002). Only mass spectral fragmentation pattern that gave greater than 90 % match were accepted.

The GC (Gas Chromatography) analysis was performed on a Shidmadzu GC 14A equipped with a FID detector using fused-silica capillary HP-5ms column (30.0 m length x 250 μm; 0.25 μm film thickness) with helium as carrier gas at a flow rate of 1 ml min^{-1}. Column temperature was programmed initially at 60 °C for 10 min, then programmed to 230 °C at 3 °C min^{-1} and held for 1 min at 230 °C.

3.9.2 Column chromatography

Column chromatography was performed using Merck Kieselgel 60 PF_{253} Art No 7734.1000 and 9385.1000 with particle size 0.063-0.200 mm and 0.040-0.0063 mm, respectively. The gel was made into slurry with solvent before it was packed onto the column and then allowed to equilibrate for at last an h before use. After the sample was introduced to the column, solvent with increasing polarity gradient was used to elute the chemical compounds from the column. Fractions collected were monitored by TLC and appropriate fractions were combined and where necessary subjected to further separation.

3.9.3 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) was routinely used to detect and separate the various compounds. The extracts from chromatography was examined by TLC using

precoated 20 x 20 cm glass plates, 0.25 mm thickness, silica gel F254 (Merck, Darmstadt, G.F.R). The TLC plates were spotted with a piece of fine glass capillary tube and then developed in saturated chromatography tanks with various solvent systems at room temperature. The spots were visualized by examination of the TLC plates under UV light (254 and/or 343 nm), followed by applying iodine vapour or spray reagent.

3.9.4 Preparative-Thin Layer Chromatography (Prep-TLC)

Preparative-Thin Layer Chromatography (prep-TLC) was used in the purification of compounds. The samples were introduced onto the plates as a continuous streak 2 cm above the base of the plates using capillary tubes. The plates were developed in a chromatographic tank saturated with the developing solvent at room temperature. The plates were then air-dried, and the desired band was scrapped out and extracted with a suitable solvent.

3.9.5 Identification of compounds in hexane extracts of *P. bleo* and *P. grandifolia* using GCMS

The hexane extracts of both *Pereskia spp.* showed strong antioxidant activities in the antioxidant screenings (Section 4.2). Thus, the hexane extracts of both *Pereskia spp.* were subjected to GCMS analysis to identify the chemical constituents in the extracts.

3.9.6 Extraction and isolation of chemical constituents from the bioactive ethyl acetate extract of *P. bleo*

Based on the results of bioactivity screenings, such as antioxidant activity (Section 4.2), antimicrobial activity (Section 4.3) and preliminary cytotoxicity screening (Section 4.4), the ethyl acetate extract of *P. bleo* was identified as the bioactive extract. The cell death elicited by the ethyl acetate extract also clearly demonstrated DNA fragmentation, indicating apoptotic cell death as the major mechanism involved (Section 4.5.2). In addition, LUX RT-qPCR analysis also showed that the apoptosis elicited by the ethyl acetate extract on KB cells was mediated largely *via* p53 although the role of caspase-3 and c-myc cannot be ruled out [Section 4.6.5 (i)]. Thus, further chemical investigation was directed to the ethyl acetate extract of *P. bleo*.

The extraction and fractionation procedures leading to the isolation of compounds is shown in Figure 3.4 and 3.5. Firstly, dried, ground leaves of *P. bleo* (1050.56 g) were extracted (3x) with methanol (1.5 L each time). The methanol-containing extract obtained was initially treated with charcoal, then filtered over Celite® and the filtrate was evaporated under reduced pressure to give a crude methanolic extract (99.44 g). Treatment with charcoal was necessary to remove the high content of chlorophyll present in the extract. The presence of chlorophyll interfered with efforts at chromatographic separation.

The crude methanol extract was then further partitioned with ethyl acetate and water using a separating funnel. The ethyl acetate-soluble layer was concentrated *in vacuo* giving an 18.34 g ethyl acetate fraction, which was subjected to flash silica gel column chromatography over Merck Kieselgel 60 (0.063-0.200 mm mesh size); elution starting with chloroform (10 L), and then with chloroform-methanol [9:1 (9 L)] and finally methanol (7.6 L). The chloroform (**X**), chloroform-methanol (**Y**) and methanol eluents (**Z**) were concentrated to give 4.89 g, 12.70 g and 3.04 g respectively, and then

subjected to cytotoxicity screening. Further purification was directed to the fraction **X** as it was found to be active in the cytotoxicity screening.

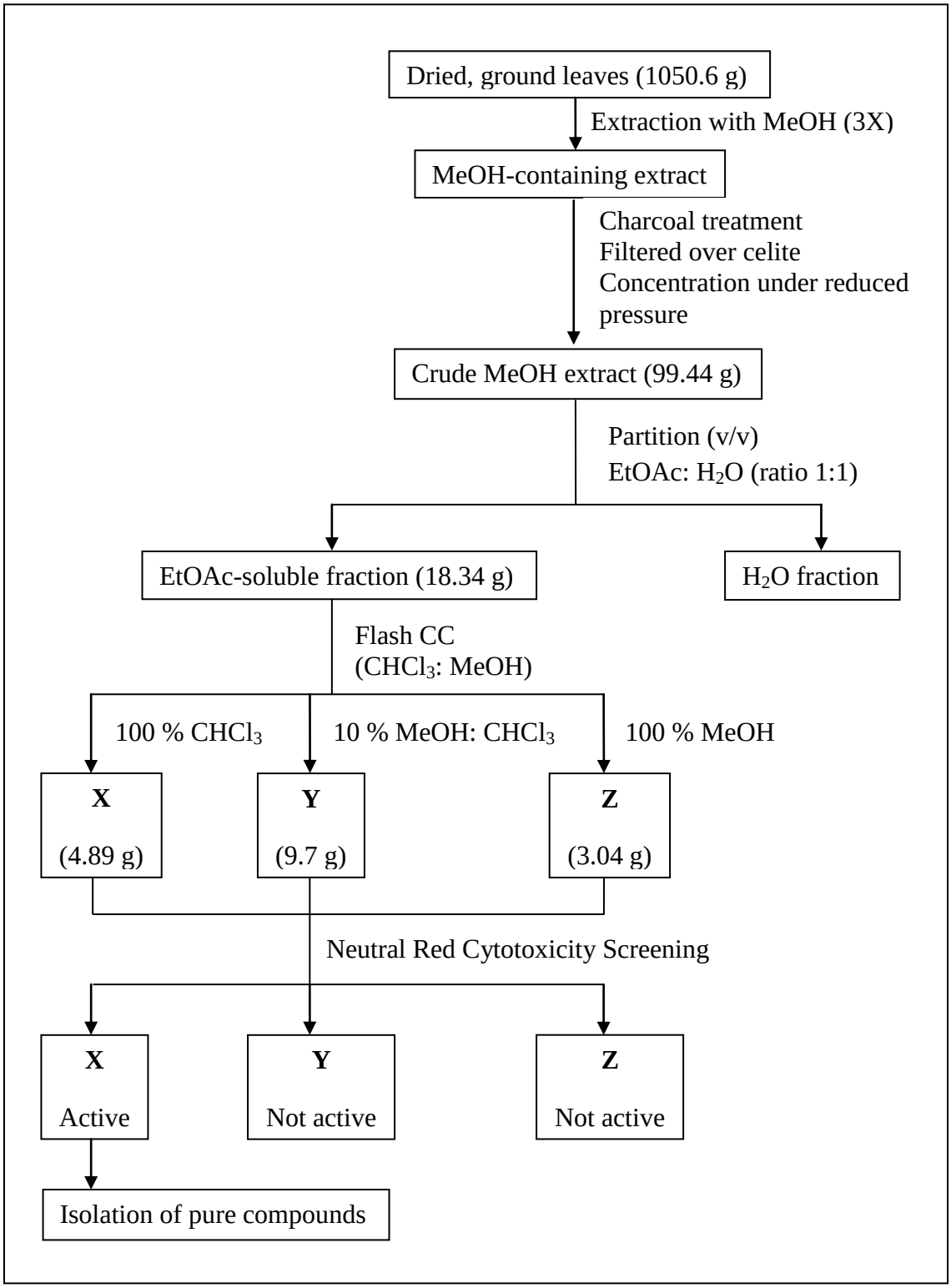
The active chloroform fraction (**X**; 3.47 g) which was a dark brown residue obtained, was then purified over a column (3 cm diameter x 49 cm height) packed with 180 g of Merck Kieselgel 60 (0.063 - 0.200 mm mesh size) and the components were separated by successive elution, initially with hexane followed by hexane enriched with increasing percentages of dichloromethane, monitoring with TLC, gave several sub-fractions (A- L).

Compound **1** (21 mg) was isolated from the sub-fraction B [dichloromethane-hexane (1:9) fraction, 192.7 mg], after further purification using prep-TLC (preparative-TLC) with ethyl acetate: hexane (1:9) as developing solvent system. Repeated prep-TLC on the sub-fraction F [dichloromethane-hexane (1.6:8.4) fraction, 64.2 mg], using chloroform as the developing solvent yielded compound **2** (10.6 mg).

Subsequently, further elution of X fraction with dichloromethane-hexane (6:4) yielded sub-fraction G (475.5 mg), which was then subjected to silica gel column chromatography over Merck Kieselgel 60 (0.063-0.200 mm size mesh size), eluting initially with hexane, followed by hexane enriched with increasing percentages of dichloromethane. Compound **3** (41.2 mg) was isolated from the fraction obtained upon elution with dichloromethane-hexane (1.6: 8.4) on silica gel column.

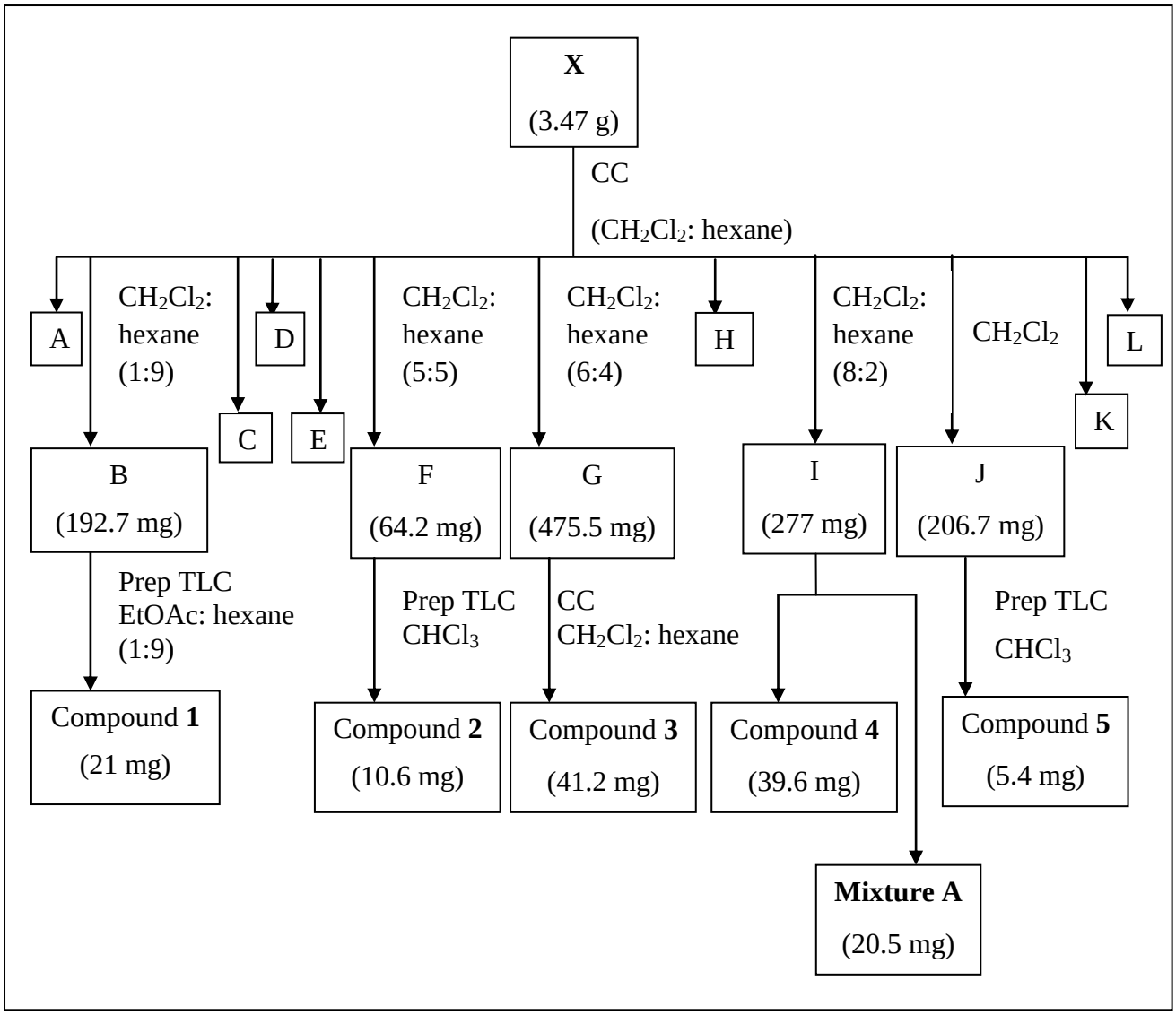
Compound **4** (39.6 mg) and **mixture A** (20.5 mg) were obtained from sub-fraction I upon elution of X fraction with dichloromethane-hexane (8: 2). Lastly, elution with dichloromethane yielded sub-fraction J (206.7 mg) containing compound **5**. Compound **5** (5.4 mg) was obtained after prep-TLC using chloroform as the developing solvent.

All the above compounds were identified using GC-MS analysis and NMR techniques (whenever necessary).



CC: column chromatography; MeOH: methanol; EtOAc: ethyl acetate; H_2O: water; $CHCl_3$: chloroform

Figure 3.4: The extraction and fractionation procedures leading to the isolation of chemical constituents from the bioactive ethyl acetate extract of *P. bleo*



CC: column chromatography; MeOH: methanol; EtOAc: ethyl acetate; H_2O: water; $CHCl_3$: chloroform; CH_2Cl_2: dichloromethane

Figure 3.5: The extraction and isolation of compounds 1-5 and mixture A from the bioactive ethyl acetate extract of *P. bleo*

3.9.7 Extraction and isolation of chemical constituents from the bioactive ethyl acetate extract of *P. grandifolia*

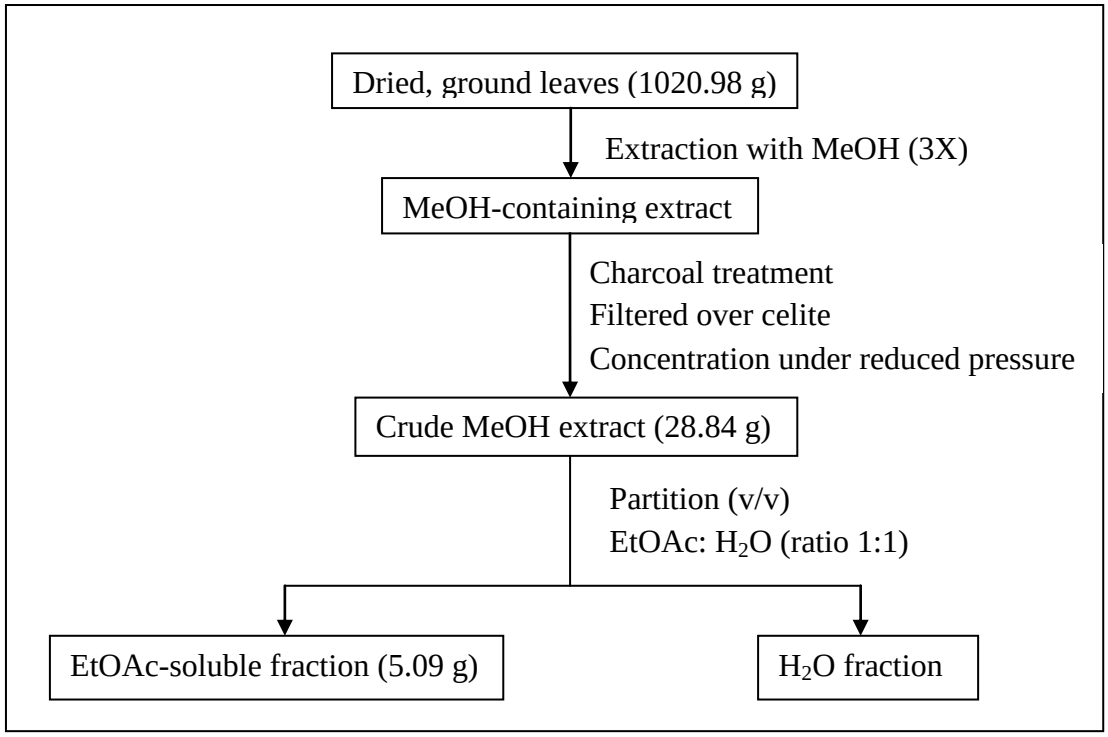
Based on the results of bioactivity screenings, such as antioxidant activity (Section 4.2), antimicrobial activity (Section 4.3) and preliminary cytotoxicity screening (Section 4.4), both the hexane and ethyl acetate extracts of *P. grandifolia* were identified as bioactive extracts. The selected cancer cell deaths treated with bioactive extracts clearly demonstrated DNA fragmentation, indicating apoptotic cell death as the major mechanism involved (Section 4.5.2). In addition, LUX RT-qPCR analysis showed that the hexane and ethyl acetate extracts killed the selected cancer cells *via* p53, caspase-3 and c-myc activation [Section 4.6.5 (ii)]. The active ingredients in hexane and ethyl acetate extracts may lead to valuable compounds. Some of the antioxidant compounds appeared in the ethyl acetate extract might also appear in the hexane extract of *P. grandifolia* or *vice versa*. Further chemical investigation was thus directed to the ethyl acetate extract of *P. grandifolia*. Fractionation into hexane and then ethyl acetate extraction was avoided as the yield of ethyl acetate extract obtained was very low (refer to Table 4.3).

The extraction and fractionation procedures leading to the isolation of compounds is shown in Figure 3.6 and 3.7. Firstly, dried and ground leaves *P. grandifolia* (1020.98 g) were extracted (3x) with methanol (1.5 L each time) at room temperature. The methanol-containing extract obtained was initially treated with charcoal, then filtered over Celite® and the filtrate was evaporated under reduced pressure to give a crude methanolic extract (28.84 g). Treatment with charcoal was necessary to remove the high content of chlorophyll present in the extract. The presence of chlorophyll interfered with efforts at chromatographic separation.

The crude methanol extract was then further partitioned with ethyl acetate and water using a separating funnel. The ethyl acetate-soluble layer was concentrated *in vacuo* giving a 5.09 g ethyl acetate fraction. The ethyl acetate fraction (4 g) was subjected to silica gel column chromatography (200 g) over Merck Kieselgel 60 (0.063-0.200 mm mesh size); initial elution with hexane followed by hexane enriched with increasing percentages of dichloromethane, monitoring with TLC, to give several main fractions: A (0.12 g), B (1.42 g), C (0.09 g), D (0.21 g), E (0.24 g) and F (1.04 g).

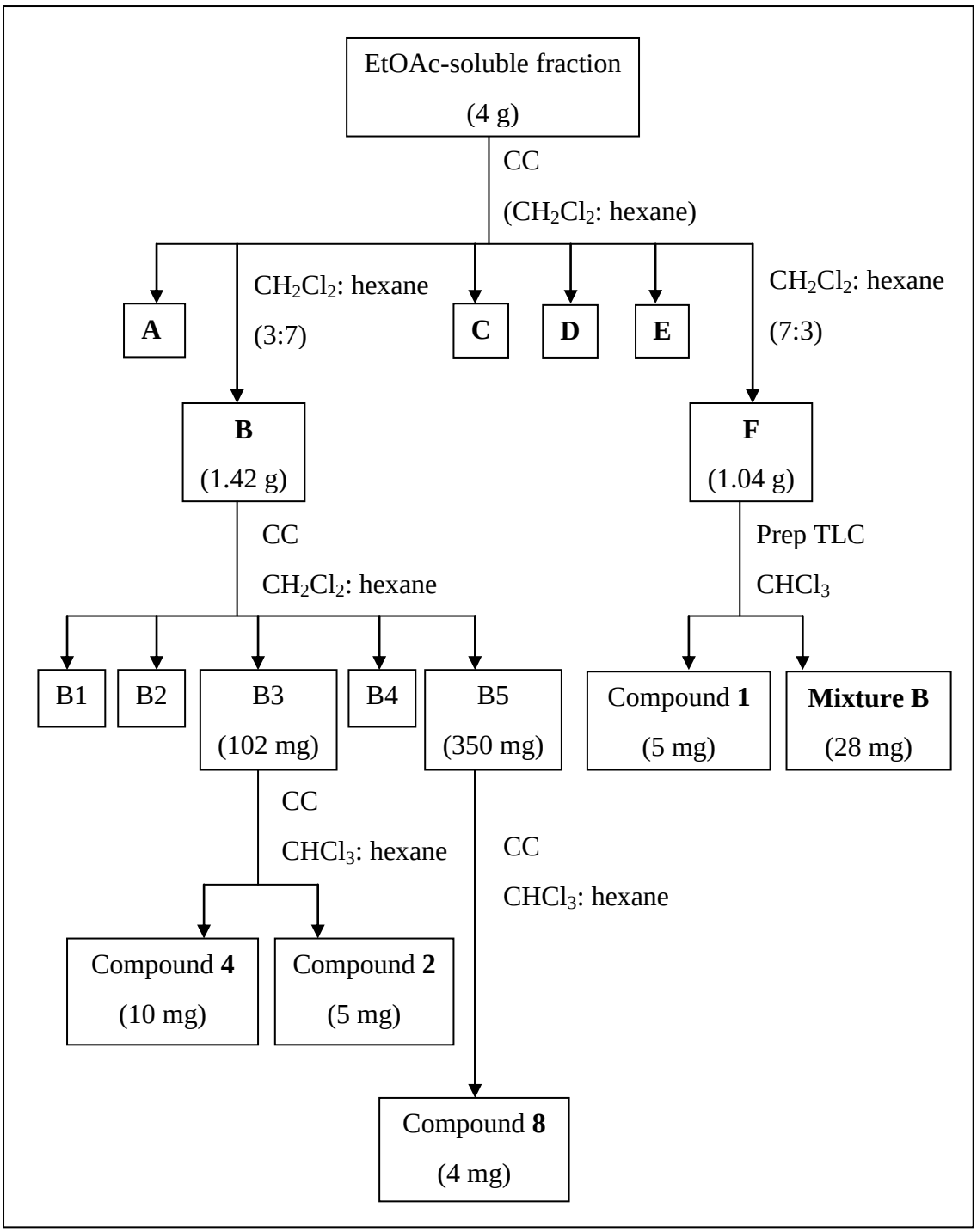
Fr. B (1.42 g) obtained was subjected to silica gel column chromatography (70 g); elution with hexane followed by hexane enriched with increasing percentages of dichloromethane gave several sub-fractions (Fr.B1-B5). Fr.B3 (102 mg) which was further subjected to silica gel column chromatography (1 g). Successive elution with hexane followed by hexane enriched with increasing percentages of chloroform gave **4** (10 mg) and **2** (5 mg). Components in Fr.B5 (350 mg) were obtained in the same manner as for Fr.B3 yielding **8** (4 mg). Repeated prep-TLC of Fr. F (1.04 g) using chloroform as developing solvent, yielded compound **1** (5 mg) and a **mixture B** (28 mg).

All the above compounds were identified using GC-MS analysis and NMR techniques (whenever necessary).



MeOH: methanol; EtOAc: ethyl acetate; H_2O: water

Figure 3.6: The extraction and fractionation procedures leading to the isolation of chemical constituents from the bioactive ethyl acetate extract of *P. grandifolia*



CC: column chromatography; MeOH: methanol; EtOAc: ethyl acetate; H_2O: water; $CHCl_3$: chloroform; CH_2Cl_2: dichloromethane

Figure 3.7: The extraction and isolation of compounds 1, 2, 4, 8 and mixture B from the bioactive ethyl acetate extract of *P. grandifolia*

3.10 Screening for alkaloids

The alkaloid screening methods were conducted according to the method of Santos *et al.* (1978) with some modification.

3.10.1 Preparation of Wagner's reagent

2.00 g of potassium iodide and 1.30 g of iodine were dissolved in enough distilled water to make 100 ml.

3.10.2 Preliminary alkaloid test

Hydrochloric acid (5 ml) was added to 20 g of methanol extract and stirred while heating for about 5 min. After the sample was cooled to room temperature, powdered sodium chloride (0.5 g) was added to the sample. The sample was then stirred and filtered. Sufficient fresh 2 M hydrochloric acid was added to wash the filtrate and bring the filtrate to a final volume of 5 ml. A few drops of Wagner's reagent were added to 1 ml of aliquot. The results were observed and recorded as follows: + slight turbidity; ++ definite turbidity and +++ heavy turbidity.

No precipitate or turbidity observed can be assumed as absence of alkaloids in the plant material. A (+), (++) or (+++) recorded was a preliminary evidence that alkaloids are present but this test must be confirmed.

3.10.3 Confirmatory test for alkaloids

Twenty eight percent (28 %) of ammonia was added drop wise to the remaining 4 ml of aliquot to render the solution alkaline to litmus. The alkaline solution was extracted three times with 10 ml chloroform each time. The combined chloroform extracts was evaporated using rotary evaporator. (The alkaline aqueous layer was reserved for the test for quaternary and / or amine oxide bases). Hydrochloric acid (2 M, 5 ml) was added to the residue with stirring over a steam bath for about two min and cooled to the room temperature. Then, it was filtered and a few drops of Wagner's reagent were added to the filtrate. The result was observed and the turbidity was recorded as (+), (++) or (+++) as in the preliminary test. A (+), (++) or (+++) recorded was indicative of the presence of a primary, secondary or tertiary alkaloids.

3.10.4 Test for quaternary and/ or amine oxide bases

Hydrochloric acid (2 M) was added drop wise to the alkaline aqueous layer above with stirring until the solution was acidic to litmus. Then, it was filtered and a few drops of Wagner's reagent were added to the filtrate. Wagner's test was repeated on the solution as in the preliminary test. A (++) or (+++) result observed in the test may be taken as an indication of the presence of quaternary and / or amine oxide bases. A (+) reaction may be the result of incomplete chloroform extraction and is considered negative for quaternary bases.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Extraction yield of *P. bleo* and *P. grandifolia*

Solvent extraction is the most popular method used in sample preparation. The yield of methanol extracts of *P. bleo* and *P. grandifolia* is shown in Table 4.1.

Table 4.1: Yield of methanol extracts of *P. bleo* and *P. grandifolia*

Plants	Samples /Extracts	Weight (g)
P. bleo	Fresh samples	4526.79
	Dried and ground plant material	752.92 (16.63 %)
	Methanol extract	79.81 (10.60 %)
P. grandifolia	Fresh samples	410.18
	Dried and ground plant material	37.05 (9.03 %)
	Methanol extract	9.91 (26.75 %)

Before extraction, the plant material needs to be dried to avoid the presence of water in the extracts. The moisture content in the leaves of *P. grandifolia* was higher than *P. bleo* as the yield of dried and ground leaves of *P. grandifolia* (9.03 %) was much lower than *P. bleo* (16.63 %). The percentage of crude methanol extract yield was based on the weight of dried and ground plant materials. The yield of crude methanol extract of *P. grandifolia* (26.75 %) was higher than *P. bleo* (10.60 %). Table 4.2 and Table 4.3 show the yield of extracts fractionated from *P. bleo* and *P. grandifolia* crude methanol extracts. Methanol was used as the extraction solvent due to its polarity and its known ability to extract compounds such as phenolics, flavonoids and other polar materials (Velioglu *et al*., 1998).

Table 4.2: Yield of extracts fractionated from *P. bleo* crude methanol extract

Extracts	Yield of extracts (g) [extracted from 33.58 g of methanol extract]	Percentage (%)
Hexane	2.19	6.52
Ethyl acetate	0.99	2.95
Water	18.14	54.02

Table 4.3: Yield of extracts fractionated from *P. grandifolia* crude methanol extract

Extracts	Yield of extracts (g) [extracted from 7.84 g of methanol extract]	Percentage (%)
Hexane	1.29	16.45
Ethyl acetate	0.56	7.14
Water	3.86	49.23

As indicated by Yan *et al.* (1999), a single solvent may not be enough to identify certain extracts responsible for the activity. The crude methanol extracts were further fractionated into hexane, ethyl acetate and water extracts. The percentage yield of fractionated extracts was based on the weight of crude methanol extract. For both *Pereskia spp*., the water fraction was the most abundant fraction (54.02 % for *P. bleo* and 49.23 % for *P. grandifolia*) among the fractionated extracts in each species while the ethyl acetate fraction gave the lowest yield which 2.95 % for *P. bleo* and 7.14 % for *P. grandifolia*.

4.2 Antioxidant activity of *P. bleo* and *P. grandifolia* extracts

Free radicals are considered as important factors in the etiology of cancer, and components with antioxidant activity have received particular attention as potential inhibitors of several cancers (Dedoussis *et al*., 2005). Various methods have been developed to measure total antioxidant activity, but none of them is an ideal reference method (Erel, 2004) and these assays are not scientifically justified (Becker *et al.*, 2004). The use of different methods is thus necessary in antioxidant activity assessment. Previous studies show that no single testing method is sufficient to estimate the antioxidant activity of a test sample (Frankel and Meyer, 2000; Huang *et al*., 2005). The combination of four methods (i.e. Folin-Ciocalteau method, scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, reducing power assay and ß-carotene method) used in this study, was suitable to evaluate the antioxidant activity of *P. bleo* and *P. grandifolia*. In general, a radical is generated in the assay, and the antioxidant response of the sample against the radical is measured (Erel, 2004). To our knowledge, there is no antioxidant study reported for both *P. bleo* and *P. grandifolia*. Antioxidant activity of *P. bleo* and *P. grandifolia* was evaluated as it had not been determined previously.

4.2.1 Determination of reducing capacity of *P. bleo* and *P. grandifolia* extracts using Folin-Ciocalteau method

The Folin-Ciocalteau reagent–based assay gained popularity and is commonly known as the total phenols (or phenolic) assay which has become a routine assay in studying phenolic antioxidants. However, a recent report by Huang *et al.* (2005) stated that the Folin-Ciocalteau Reagent actually measures a sample's reducing capacity, but this is not reflected in the name 'total phenol assay'. The exact chemical nature of the

Folin-Ciocalteau reagent is not known, but it is believed to contain heteropoly-phosphotunstates-molybdates. Sequences of reversible one- or two- electron reduction reactions lead to blue species, possibly $(PMoW_{11}O_{40})^{4-}$. Basically, it is believed that the molybdenum is easier to be reduced in the complex (Huang *et al*., 2005). The electron-transfer reaction occurs between reductants and Mo(VI) is as shown as below:

Mo(VI)	+ e ⟶	Mo(V)
(yellow)		(blue)

The Folin-Ciocalteau reagent is not specific to phenolic compounds as it can also be reduced by many non-phenolic compounds [e.g. vitamin C and Cu(I)]. Phenolic compounds react with Folin-Ciocalteau reagent only under basic conditions (adjusted by a sodium carbonate solution to pH ~10). Dissociation of a phenolic proton leads to a phenolate anion, which is capable of reducing Folin-Ciocalteau reagent. This supports the notion that the reaction occurs through electron-transfer mechanism. The blue compounds formed between phenolate and Folin-Ciocalteau reagent are independent of the structure of phenolic compounds, therefore ruling out the possibility of coordination complexes formed between the metal center and the phenolic compounds (Huang *et al*., 2005).

The reducing capacity of *P. bleo* and *P. grandifolia* extracts, determined from regression quotation of calibration curve ($y=0.0012x+0.0004$, $R^2 = 0.9931$, Figure 4.1) were expressed as mg of GAEs per gram of methanol or fractionated extracts. The absorbance value of the test extract after subtraction of control was translated into reducing capacity [mg/l of gallic acid equivalents (GAEs)] using the gallic acid calibration plot with the following formula:

$$\text{Reducing capacity (mg/l of GAEs)} = \frac{(y - 0.0004)}{0.0012}$$

The reducing capacity of *P. bleo* and *P. grandifolia* extracts are shown in Table 4.4. BHA was used as positive reference standard in the study.

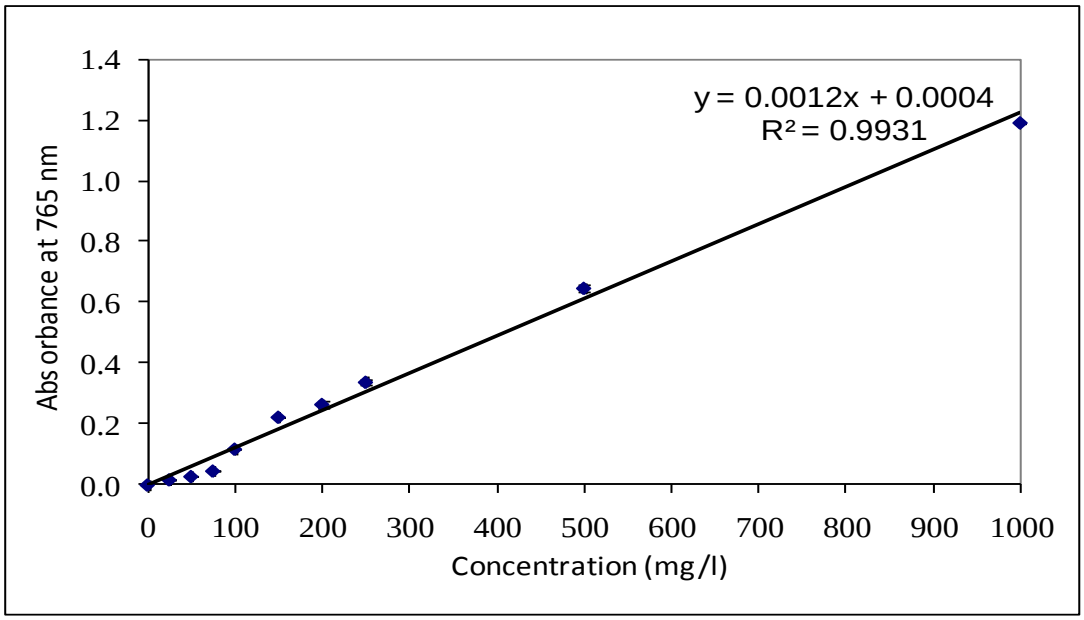


Figure 4.1: The gallic acid calibration graph

Table 4.4: Reducing capacity of *P. bleo* and *P. grandifolia* extracts

Plants / Standard	Extracts	Reducing capacity (mg of GAEs/g of methanol or fractionated extracts)
P. bleo	Methanol	27.88 ± 0.28 [a]
	Hexane	23.15 ± 0.35 [a]
	Ethyl acetate	40.12 ± 0.66 [b]
	Water	27.70 ± 0.18 [a]
P. grandifolia	Methanol	38.54 ± 0.48 [b]
	Hexane	19.08 ± 0.43 [a]
	Ethyl acetate	45.99 ± 0.30 [b]
	Water	35.79 ± 0.33 [b]
BHA (positive reference standard)		252.97 ± 2.81

GAEs, gallic acid equivalents

Values expressed are mean ± standard deviation of three measurements. Means with different letters in the same column for each species are significantly different ($p < 0.05$, ANOVA).

Generally, the *P. grandifolia* showed significantly higher reducing capacity than *P. bleo*. The highest amount was found in the ethyl acetate extract of *P. grandifolia* with 45.99 mg of GAEs/g of extract. For both *Pereskia spp*., the reducing capacity in the ethyl acetate extracts were the highest (40.12 mg of GAEs/g of extract for *P. bleo* and 45.99 mg of GAEs/g of extract for *P. grandifolia*), followed by the methanol extracts, water extracts and hexane extracts, although the yield of ethyl acetate extracts were the lowest among the fractionated extracts for both *Pereskia spp*. (Table 4.2 and Table 4.3). The significantly higher reducing capacity in the ethyl acetate extracts than in the crude methanol extracts was probably due to the purification and concentration of chemical constituents throughout the fractionation procedure. The high reducing capacity of ethyl acetate for both plant samples might contribute toward their antioxidant and related activity. The methanol, hexane and water extracts of *P. bleo* had similar amount of reducing capacity ($p < 0.05$) while the reducing capacity of methanol and water extracts of *P. grandifolia* were similar ($p < 0.05$). Figure 4.2 shows the comparison of reducing capacity (mg of GAEs/g of extract) of both *Pereskia spp.*

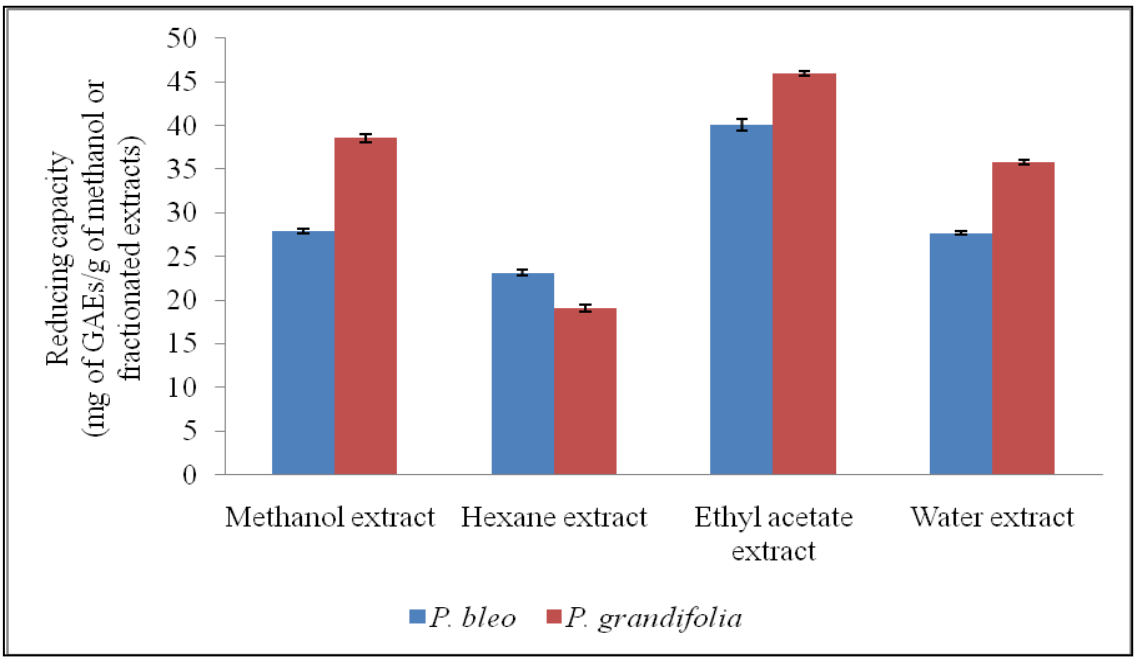


Figure 4.2: Reducing capacity of *P. bleo* and *P. grandifolia* extracts

4.2.2 Scavenging activity of *P. bleo* and *P. grandifolia* extracts on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals

Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. The method of scavenging DPPH free radicals can be used to evaluate the antioxidant activity of specific compounds or extract in a short time (Cheung *et al*., 2003). This method is sensitive and only requires small amount of samples (Blois, 1958) and allows testing of both lipophilic and hydrophobic substances (Kulisic *et al*., 2004). The proton radical-scavenging action is known to be one of the various mechanisms for antioxidation.

The DPPH assay was always believed to involve hydrogen atom transfer reaction, however, a recent paper by Foti *et al.* (2004) suggested otherwise. On the basis of the kinetic analysis of the reaction between phenols and DPPH, Foti and co-workers (2004) suggested that the reaction in fact behaves like an electron transfer reaction. The authors found that the rate-determining step for this reaction consist of a fast electron-transfer process from the phenoxide anions to DPPH. The hydrogen atom abstraction from the neutral ArOH by DPPH becomes a marginal reaction path, because it occurs very slowly in strong hydrogen-bond-accepting solvents, such as methanol and ethanol (Huang *et al*., 2005).

DPPH radical scavenging activity is determined by a colorimetric assay. DPPH is a nitrogen-centred free radical, stable at room temperature, and produces a purple solution in methanol. The reducing capability of the DPPH radical is determined by the decrease in its absorbance induced by antioxidants. The decrease in absorbance of DPPH radical caused by antioxidants is due to the reaction between antioxidant molecules and radical, which results in the scavenging of the radical by electron transfer (Figure 4.3). This is visualized as a discolouration from purple to yellow.

(Gülçin *et al*., 2004; Duh *et al*., 1999; Chang *et al*., 2002; Gülçin *et al*., 2003). The principal of DPPH radical scavenging assay is shown in Figure 4.3.

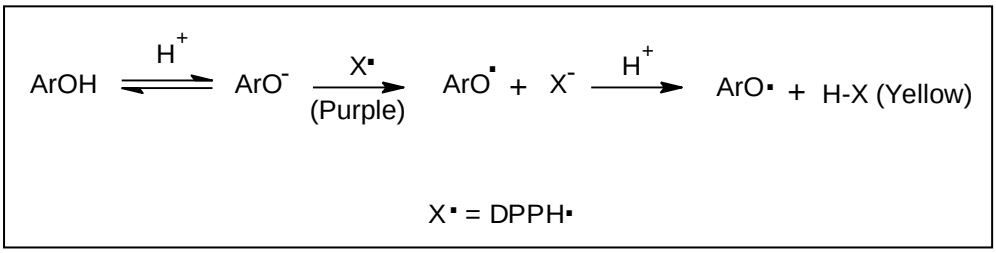


Figure 4.3: The principal of DPPH radical scavenging assay (Foti *et al*., 2004)

Extracts of *P. bleo* and *P. grandifolia* contained active substances that were capable of scavenging DPPH radicals (Figure 4.4 and 4.5). In the DPPH scavenging assay, extracts of *P. bleo* and *P. grandifolia* were investigated through the free radical scavenging activity *via* their reaction with the stable DPPH radicals. The radical scavenging effects of both *Pereskia spp*. extracts and positive reference standards on DPPH are shown in Figure 4.4, Figure 4.5 and Figure 4.6. Scavenging effects of extracts on DPPH radicals increased with increased concentrations of extracts. Table 4.5 shows the scavenging activity (IC_{50} values) of extracts on the inhibition of scavenging activity of DPPH radicals.

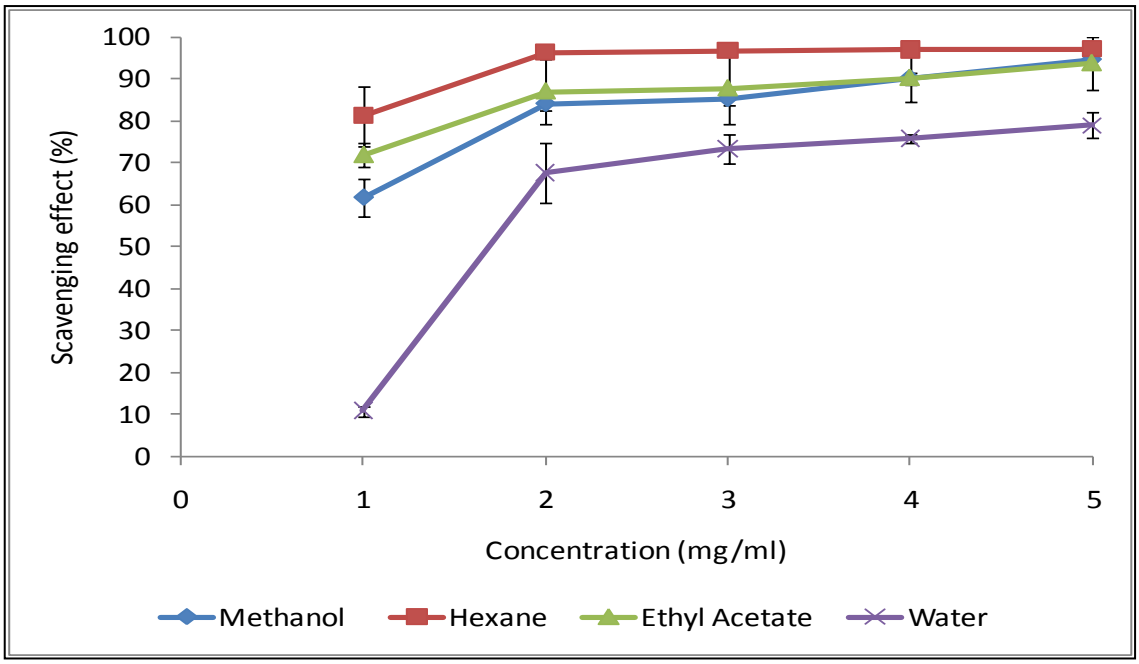


Figure 4.4: Scavenging effect of *P. bleo* extracts on DPPH radical. Each value is expressed as mean ± standard deviation of three measurements.

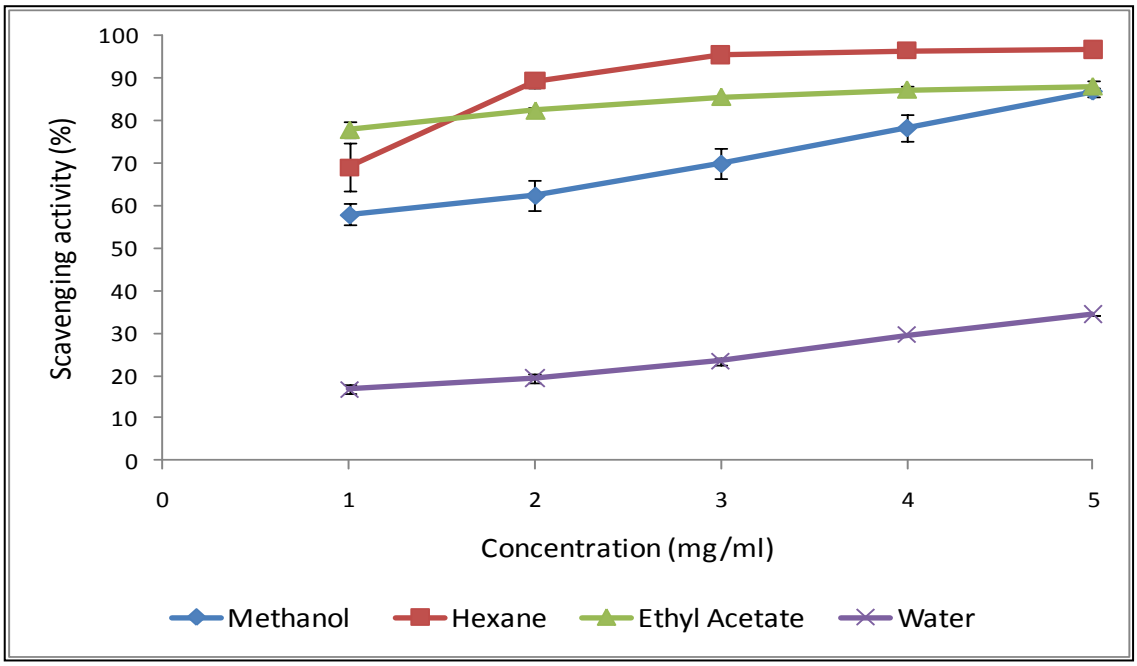


Figure 4.5: Scavenging effect of *P. grandifolia* extracts on DPPH radical. Each value is expressed as mean ± standard deviation of three measurements.

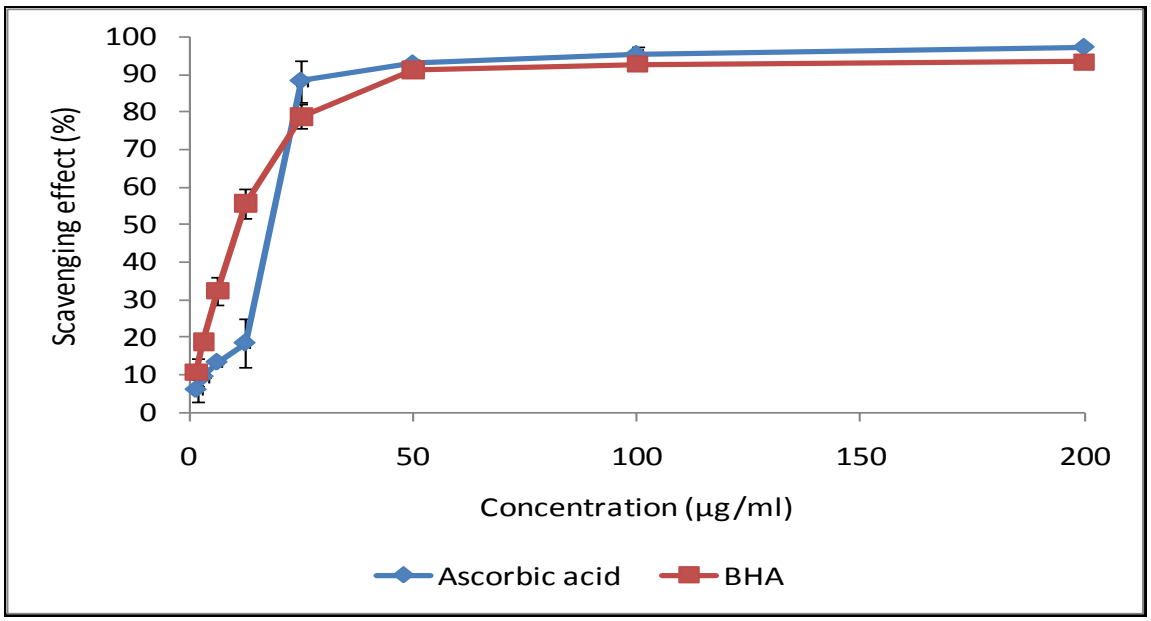


Figure 4.6: Scavenging effect of positive reference standards (BHA and ascorbic acid) on DPPH radical. Each value is expressed as mean ± standard deviation of three measurements.

Table 4.5: The scavenging activity (IC_{50} values) of *P. bleo* and *P. grandifolia* extracts on the inhibition of scavenging activity of DPPH radicals

Plants / Standards	Extracts	IC_{50} values (mg/ml)	IC_{50} values (µg/ml)
P. bleo	Methanol	< 1	750
	Hexane	< 1	210
	Ethyl Acetate	< 1	225
	Water	1.70	-
P. grandifolia	Methanol	< 1	860
	Hexane	< 1	285
	Ethyl Acetate	< 1	140
	Water	> 5	-
Positive reference standard	Ascorbic acid	-	19
	BHA	-	11

The *P. grandifolia* water extract with IC_{50} value of > 5 mg/ml, was not effective in scavenging DPPH radical while the *P. bleo* water extract expressed considerable scavenging effect (IC_{50} 1.70 mg/ml). Extracts that possessed IC_{50} values less than 1.0

mg/ml were retested for their scavenging activity on more intensive concentration (Table 3.3) to determine the IC_{50} values (Figure 4.7 and Figure 4.8). The ethyl acetate extract of *P. grandifolia* showed the best DPPH scavenger with the lowest IC_{50} 140 µg/ml, followed by the hexane and ethyl acetate extracts of *P. bleo* with IC_{50} values of 210 and 225 µg/ml respectively. For both *Pereskia spp.*, the stronger scavenging activity in the hexane and ethyl acetate fractions than in the crude methanol extracts was probably due to the concentration of antioxidant compounds in the respective extracts.

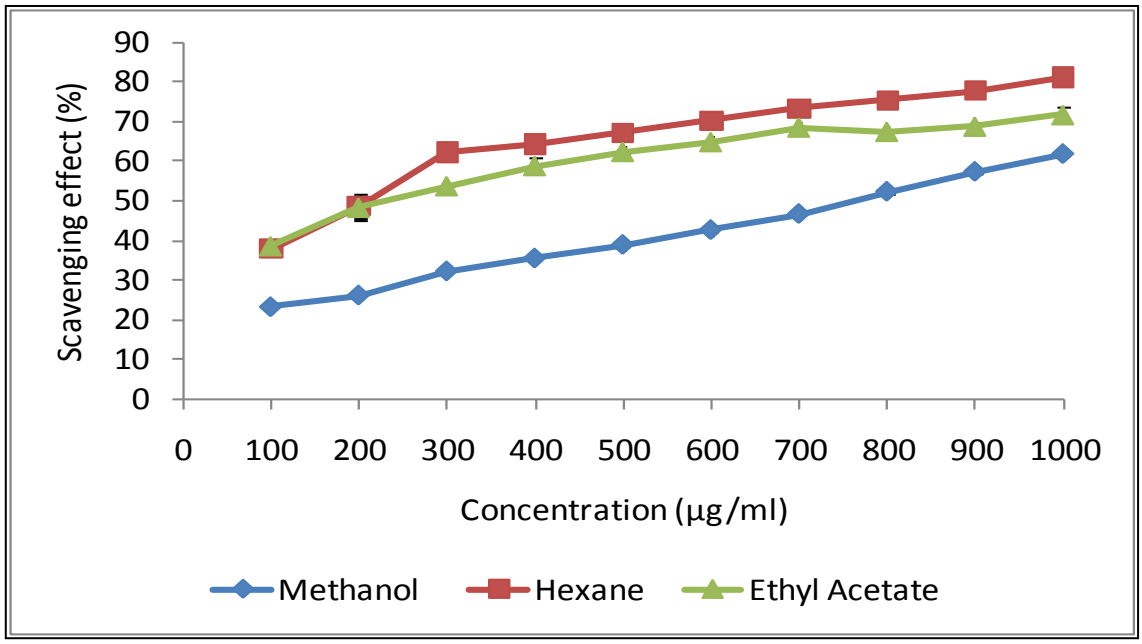


Figure 4.7: Scavenging effect of lower concentrations of *P. bleo* extracts on DPPH radical to determine the IC_{50} values. Each value is expressed as mean ± standard deviation of three measurements.

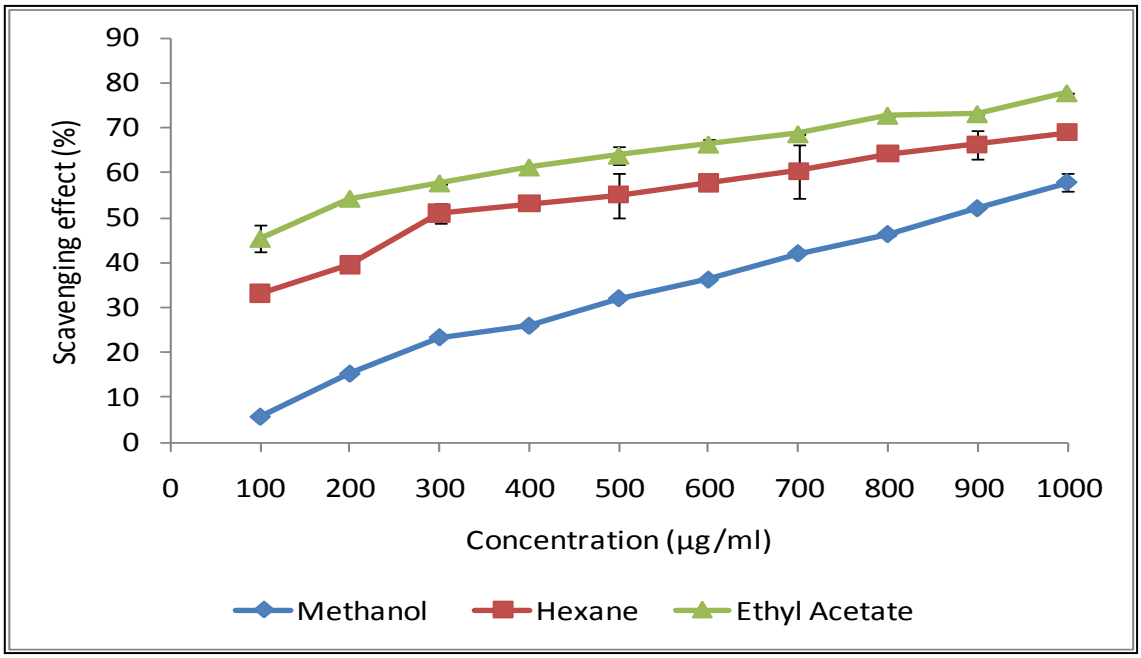


Figure 4.8: Scavenging effect of lower concentrations of *P. grandifolia* extracts on DPPH radical to determine the IC_{50} values. Each value is expressed as mean ± standard deviation of three measurements.

Generally, in comparison to both positive reference standards, which were ascorbic acid (IC_{50} 19 µg/ml) and BHA (IC_{50} 11 µg/ml), all the extracts displayed weak scavenging activity (Table 4.5). These results revealed that extracts of both *Pereskia spp*., except water extract of *P. grandifolia*, were free radical inhibitors or scavengers, acting possibly as primary antioxidants. The extracts might react with free radicals, particularly peroxy radicals, which are the major propagators of the autoxidation chain of fat, thereby terminating the chain reaction (Mau *et al*., 2002). According to Huang *et al.* (2005), many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH.

The Folin-Ciocalteu method, generally measures the total reducing capacity of a sample; thus results obtained generally correlate with redox and antioxidants capacities measured by other electron transfer-based antioxidant capacity assay [e.g. DPPH, Ferric ion reducing antioxidant parameter (FRAP) and Trolox equivalent antioxidant

capacity (TEAC)]. This is not surprising due to the similarity of chemistry between the assays.

4.2.3 Reducing power assay of *P. bleo* and *P. grandifolia* extracts

Different studies have indicated that the antioxidant effect is related to the development of reductones (Duan *et al.*, 2006; Dorman *et al*., 2003 and Shon *et al*., 2003) as reductones were reported to be terminators of free radical chain reactions. Thus the antioxidant activity of both *Pereskia spp.* extracts and fractions may be related to their reductive activity. In this assay, the yellow colour of the test solutions change to various shades of green and blue depending upon the reducing power of each extract. The presence of reductants (i.e. antioxidants) in the extract would result in the reduction of the Fe^{3+}/ ferric cyanide complex to the ferrous form (Fe^{2+}) as shown below:

Fe^{3+}	+	e	⟶	Fe^{2+}
(yellow)		(from antioxidant)		(blue)

The Fe^{2+} ions can therefore be monitored by measuring the formation of Pearl's Prussian blue at 700 nm. Increased absorbance at 700 nm indicates an increase in reducing power of test samples (Lih *et al*., 2001).

Ascorbic acid and BHA which were used as positive reference standards in the present study showed significantly high reducing power ($p < 0.05$), which indicated high UV absorption (2.343- 2.616) at wavelength 700 nm. The reducing powers of ascorbic acid and BHA at 5 mg/ml were 2.343 and 2.432, respectively and increased to 2.579 and 2.616, respectively at 20 mg/ml. The reducing power of *P. bleo* extracts, *P. grandifolia* extracts and positive reference standards are shown in Table 4.6 and Figure 4.9-4.11.

The reducing power of all *Pereskia spp.* extracts varied significantly with different concentrations ($p < 0.05$; Table 4.6). The reducing power of all the extracts gradually increased with increasing concentration of the extracts. This indicated that all of the extracts possessed the ability (either strong or weak) to reduce Fe^{3+}/ ferric cyanide complex to the ferrous form when evaluated by reducing power assay. However, the reducing power of the positive reference standards (ascorbic acid and BHA) were relatively more pronounced than the tested extracts.

Hexane extract showed significantly ($p < 0.05$) the highest reducing activity among the extracts of *P. bleo* with 1.633 at 5 mg/ml, 1.846 at 10 mg/ml and 2.072 at 15 mg/ml and 2.222 at 20 mg/ml (Table 4.6). The water and methanol extracts possessed some considerable reducing activity compared to the positive reference standards while the ethyl acetate extract showed the lowest ($p < 0.05$) reducing power among the extracts of *P. bleo* (Figure 4.10).

Among the *P. grandifolia* extracts, methanol and hexane extracts appeared to possess significantly ($p < 0.05$) the highest reducing activity, in comparison to ascorbic acid and BHA as reference compounds (Figure 4.11). Reducing power of methanol extract of *P. grandifolia* were 1.056, 1.946, 2.204 and 2.460 when tested at concentrations of 5, 10, 15 and 20 mg/ml respectively while reducing power of hexane extract of *P. grandifolia* was 1.557 at 5 mg/ml and 2.015 at 20 mg/ml (Table 4.6). The reducing power of water extract of *P. grandifolia* was significantly ($p < 0.05$) the lowest at 0.168, 0.227, 0.350 and 0.466 when tested at concentrations of 5, 10, 15 and 20 mg/ml respectively among all the extracts for both *Pereskia spp.* The reducing power of the methanol extract 2.460 at 20 mg/ml were comparable to that of ascorbic acid and BHA at 15 mg/ml.

For both *Pereskia spp*., the stronger reducing power in the hexane extracts than other fractionated extracts was probably due to the concentration of antioxidant compounds in the extract. The results presented here revealed that the hexane extracts of *P. bleo* and *P. grandifolia* are electron donors and might contained higher amounts of reductones, which could react with free radicals, converting them to more stable products, and terminating radical chain reaction. The hexane extracts of both *Pereskia spp.* were found to contain mainly methyl esters. It is highly probable that the lone pairs of electron on the carbonyl oxygen can be easily donated to the ferric ions in the reducing power assay. Methyl palmitate, methyl linoleate, methyl α-linolenate and phytol were identified from the hexane extract of *P. bleo* by GCMS analysis whilst methyl palmitate, methyl linoleate, methyl α-linolenate and methyl stearate were identified from the hexane extract of *P. grandifolia* (section 4.8.1 and 4.8.2).

Table 4.6: Reducing powers of extracts of *P. bleo* and *P. grandifolia* at various concentrations

Plants / Standards	Extracts	Concentrations of extracts (mg/ml)			
		5	10	15	20
P. bleo	Methanol	0.645 ± 0.03 ^{awi}	1.100 ± 0.05 ^{bxi}	1.465 ± 0.03 ^{cxi}	1.605 ± 0.04 ^{dxi}
	Hexane	1.633 ± 0.02 ^{axj}	1.846 ± 0.03 ^{byj}	2.072 ± 0.06 ^{czj}	2.222 ± 0.04 ^{dzj}
	Ethyl acetate	0.665 ± 0.00 ^{awj}	0.890 ± 0.07 ^{bwi}	1.009 ± 0.00 ^{cwi}	1.155 ± 0.05 ^{dwi}
	Water	0.670 ± 0.06 ^{awj}	1.175 ± 0.02 ^{bxj}	1.676 ± 0.05 ^{cyj}	1.834 ± 0.03 ^{dyj}
P. grandifolia	Methanol	1.056 ± 0.05 ^{ayj}	1.946 ± 0.08 ^{bzj}	2.204 ± 0.10 ^{czj}	2.460 ± 0.00 ^{dzj}
	Hexane	1.557 ± 0.02 ^{azi}	1.827 ± 0.03 ^{byi}	2.009 ± 0.03 ^{cyi}	2.015 ± 0.01 ^{cyi}
	Ethyl acetate	0.664 ± 0.00 ^{axi}	0.952 ± 0.04 ^{bxj}	1.343 ± 0.14 ^{cxj}	1.560 ± 0.06 ^{dxj}
	Water	0.168 ± 0.00 ^{awi}	0.227 ± 0.01 ^{bwi}	0.350 ± 0.01 ^{cwi}	0.466 ± 0.01 ^{dwi}
Positive reference standard	Ascorbic acid	2.343 ± 0.05 ^{a}	2.451 ± 0.02 ^{b}	2.496 ± 0.02 ^{b}	2.579 ± 0.04 ^{c}
	BHA	2.432 ± 0.01 ^{a}	2.458 ± 0.03 ^{a}	2.549 ± 0.02 ^{b}	2.616 ± 0.02 ^{c}

Absorbance values expressed are mean ± standard deviation of triplicate measurements.

For the same extract or standard with different concentrations, means in the same row with different letters (a-d) were significantly different ($p < 0.05$, ANOVA). For different species with the same concentration and same extraction solvent, means with different letters (i,j) were significantly different ($p < 0.05$, ANOVA). For the same species with the same concentration but different extraction solvent, means with different letters (w-z) were significantly different ($p < 0.05$, ANOVA).

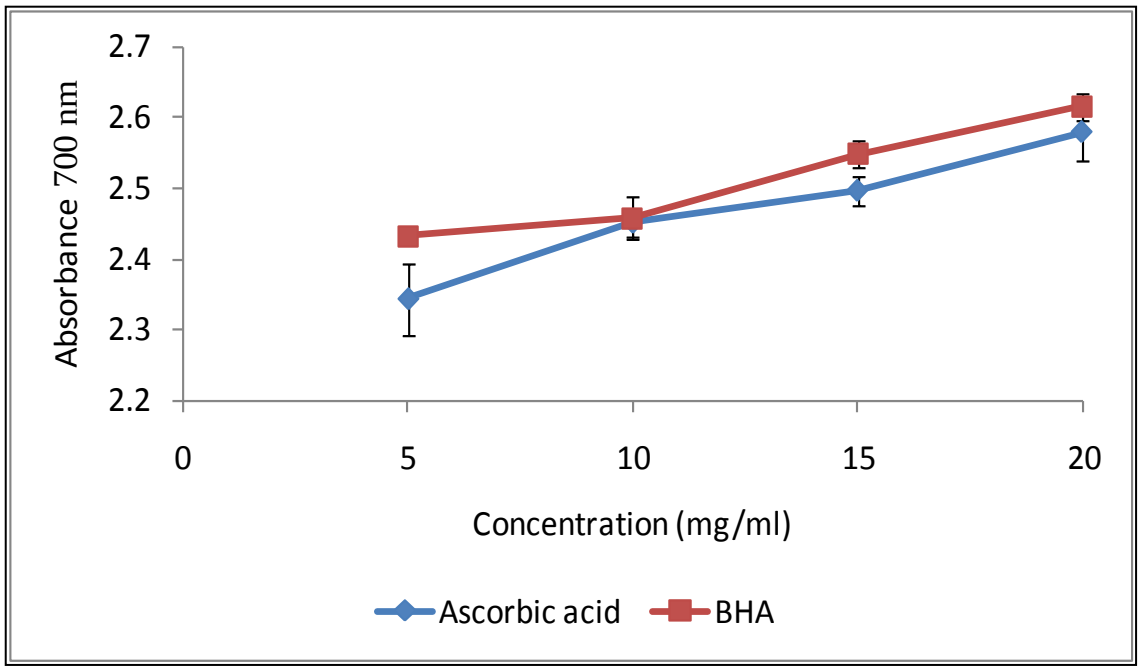


Figure 4.9: Reducing powers of ascorbic acid and BHA (reference compounds). Each value is expressed as mean ± standard deviation.

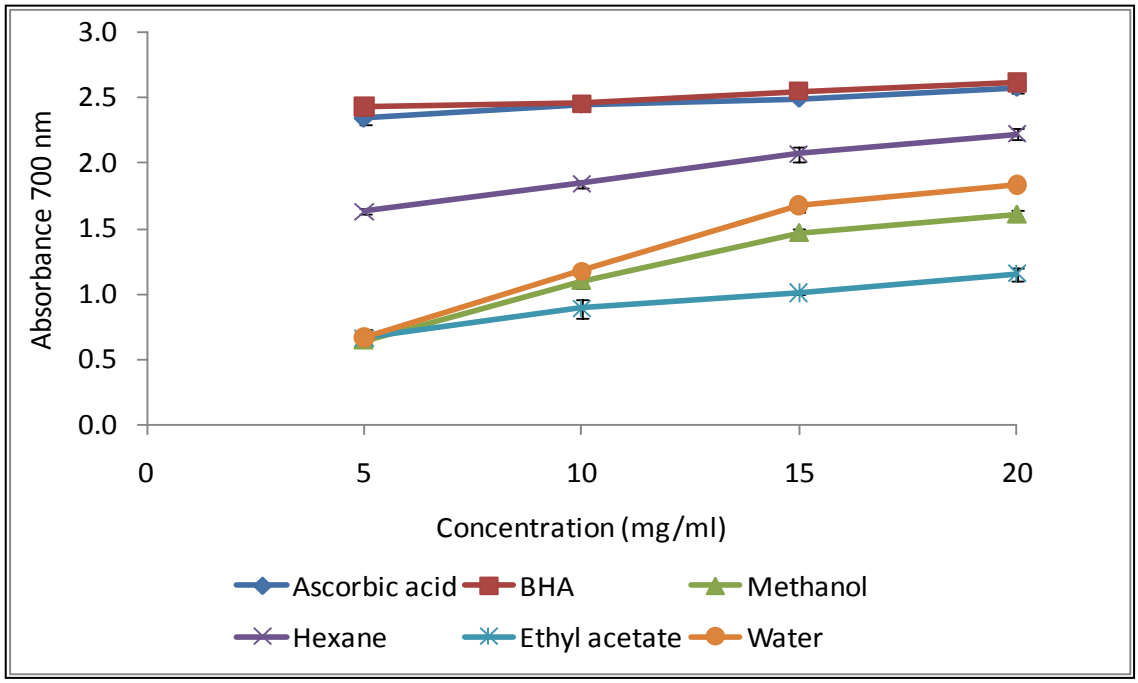


Figure 4.10: Reducing powers of extracts of *P. bleo* at various concentrations. Each value is expressed as mean ± standard deviation.

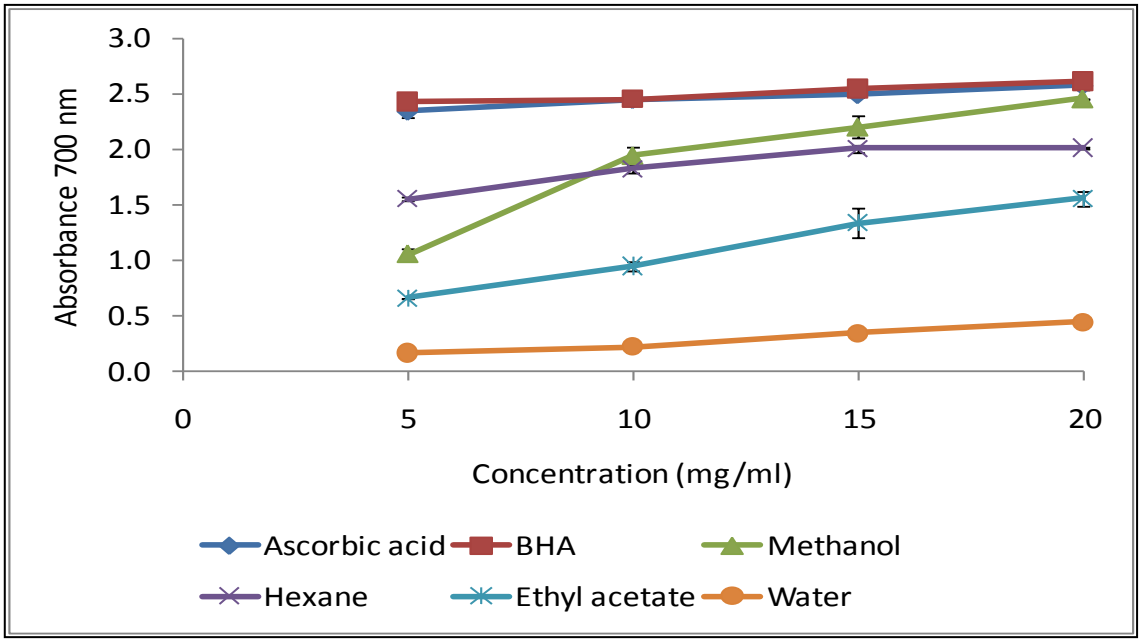


Figure 4.11: Reducing powers of extracts of *P. grandifolia* at various concentrations. Each value is expressed as mean ± standard deviation.

4.2.4 ß-Carotene bleaching activity of *P. bleo* and *P. grandifolia* extracts

ß-Carotene in this model system undergoes rapid discoloration in the absence of an antioxidant. The mechanism of bleaching of ß-carotene is a free-radical-mediated phenomenon resulting from the hydroperoxides formed from linoleic acid by air oxidation. The antioxidant activity of carotenoids is based on the radical adducts of carotenoids with free radical formed from linoleic acid. The linoleic acid free radical, formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups, attacks the highly unsaturated ß-carotene molecules. As a result, ß-carotene molecules lose their double bonds by oxidation in this model system. In the absence of an antioxidant, the ß-carotene molecule loses its chromophore and characteristic orange colour, which can be monitored spectrophotometrically. The presence of different antioxidants can hinder the extent of ß-carotene bleaching by neutralizing the linoleate-free radical and other free radicals form in the system (Jayaprakasha *et al*., 2001; Wong

et al., 2009; Kumaran and Joel Karunakaran, 2006). Thus, this forms the basis by which *Pereskia* extracts can be screened for their antioxidant potential.

Table 4.7 shows the antioxidant activities of the *Pereskia* extracts and BHA with the coupled oxidation of ß-carotene and linoleic acid. The antioxidant activities of the extracts for both *Pereskia spp.* varied significantly with different concentrations ($p < 0.05$; Table 4.7, Figure 4.12 and Figure 4.13). The ß-carotene bleaching activity of all the extracts gradually increased with increasing concentration of the extracts.

For both *P. bleo* and *P. grandifolia*, the water extracts showed significantly ($p < 0.05$) the lowest antioxidant activity while the ethyl acetate extracts presented significantly ($p < 0.05$) the highest activity (at all concentrations tested). This indicated that compounds with the strongest antioxidant activity in the ß-carotene-linoleate assay system were also of medium polarity.

The ethyl acetate extracts of *P. bleo* and *P. grandifolia* exhibited 83.68 % and 83.13 % antioxidant activity respectively at 20 mg/ml which was nearly comparable to that of BHA standard at 20 mg/ml (92.46 %; Table 4.7). It is probable that the antioxidative components in the *Pereskia* extracts can reduce the extent of ß-carotene destruction by neutralizing the linoleate free radical and other free radicals formed in the system.

Table 4.7: Antioxidant activity (%) of *P. bleo* and *P. grandifolia* extracts measured by ß-carotene bleaching method

Plants / Standard	Extracts	Concentrations of extracts (mg/ml)			
		4	8	16	20
P. bleo	Methanol	34.62 ± 0.98 ^{axj}	37.90 ± 0.62 ^{awi}	64.03 ± 2.96 ^{bxyj}	72.96 ± 2.78 ^{cxi}
	Hexane	40.57 ± 1.15 ^{axyi}	46.11 ± 1.17 ^{bxi}	62.38 ± 4.19 ^{cxi}	73.31 ± 1.60 ^{dxj}
	Ethyl acetate	42.21 ± 4.05 ^{ayi}	52.87 ± 4.71 ^{byi}	69.92 ± 3.22 ^{cyi}	83.68 ± 0.57 ^{dyi}
	Water	27.97 ± 5.24 ^{awj}	36.84 ± 2.45 ^{bwi}	48.38 ± 2.27 ^{cwi}	46.17 ± 1.56 ^{cwi}
P. grandifolia	Methanol	17.83 ± 0.94 ^{awi}	43.13 ± 3.58 ^{bwi}	56.21 ± 2.63 ^{cwi}	74.48 ± 1.94 ^{dyi}
	Hexane	40.97 ± 0.64 ^{axi}	53.64 ± 0.75 ^{bwj}	60.54 ± 2.08 ^{cwi}	65.51 ± 1.76 ^{dxi}
	Ethyl acetate	55.04 ± 3.12 ^{ayj}	58.53 ± 1.52 ^{axi}	68.68 ± 3.47 ^{bxi}	83.13 ± 3.99 ^{czi}
	Water	15.66 ± 2.88 ^{awi}	39.22 ± 2.52 ^{byi}	55.68 ± 1.96 ^{cwj}	55.60 ± 0.82 ^{cwj}
BHA (Positive reference standard)		70.80 ±1.09 ^{a}	79.00 ± 0.52 ^{b}	88.56 ± 0.82 ^{c}	92.46 ± 2.52 ^{d}

Values expressed are mean ± standard deviation of triplicate measurements.

For the same extract or standard with different concentrations, means in the same row with different letters (a-d) were significantly different ($p < 0.05$, ANOVA). For different species with the same concentration and same extraction solvent, means with different letters (i,j) were significantly different ($p < 0.05$, ANOVA). For the same species with the same concentration but different extraction solvent, means with different letters (w-z) were significantly different ($p < 0.05$, ANOVA).

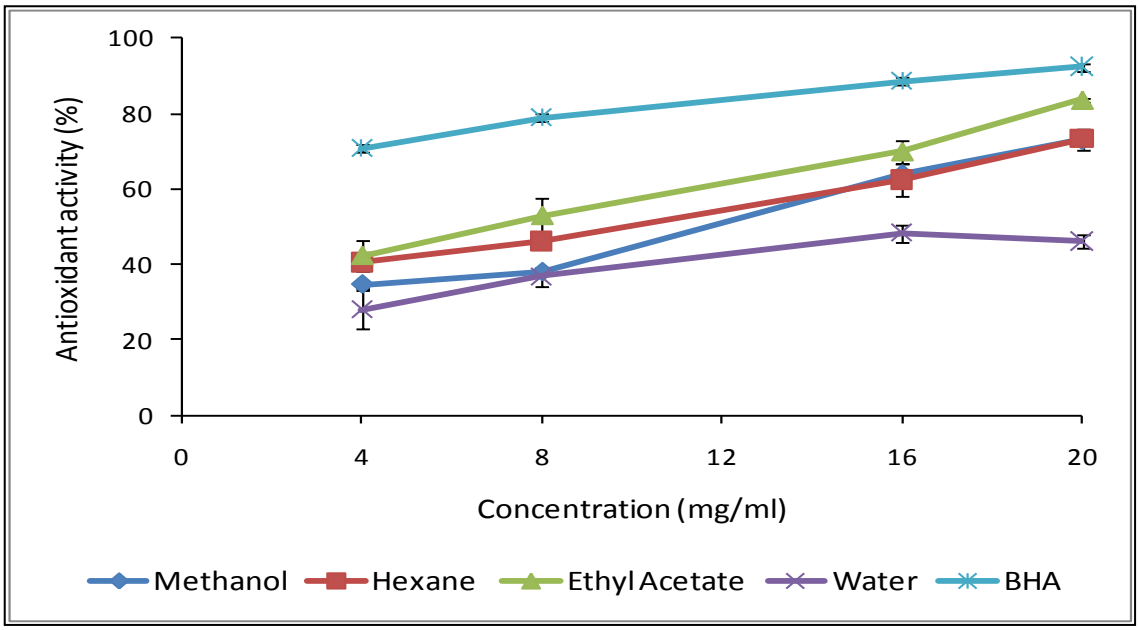


Figure 4.12: Antioxidant activity (%) of *P. bleo* extracts measured by ß-carotene bleaching method. Each value is expressed as mean ± standard deviation.

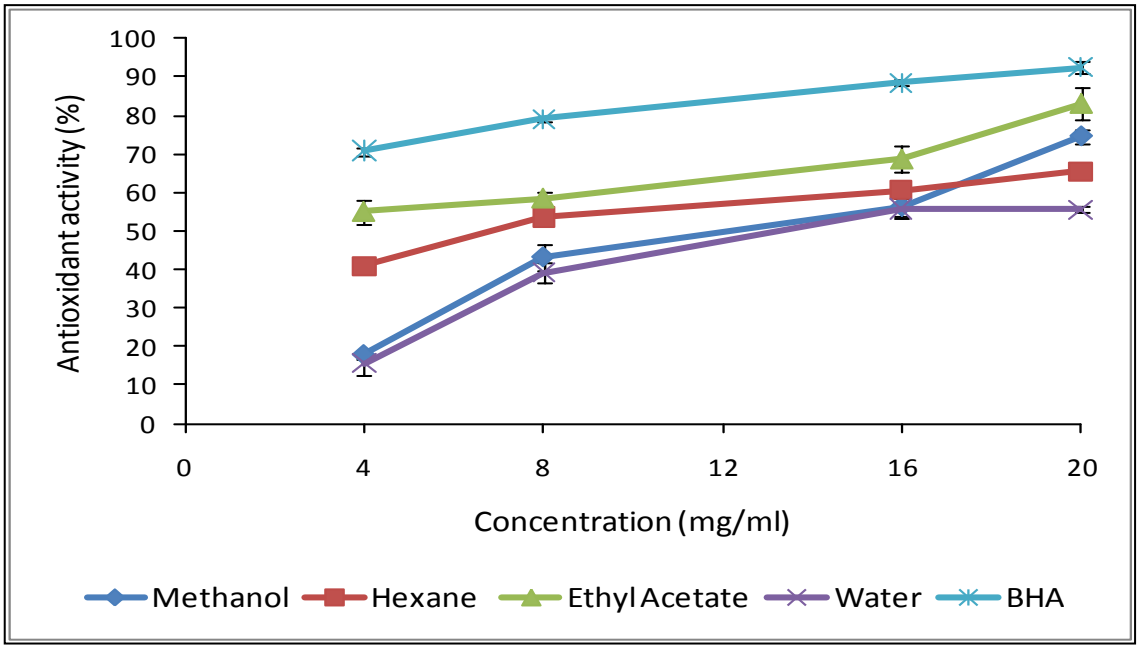


Figure 4.13: Antioxidant activity (%) of *P. grandifolia* extracts measured by ß-carotene bleaching method. Each value is expressed as mean ± standard deviation.

4.2.5 Comparison of antioxidant activity of *P. bleo* and *P. grandifolia* extracts

The differences in each antioxidant activity detection system lie in the unique characteristic of each test. This explains why employment of different method to measure antioxidant activity with various mechanisms may lead to different observations. Thus, it is not possible to make an absolute comparison of different methods. There are several methodological limitations for antioxidant determinations. The possible synergistic action among the different components present in the extracts cannot currently be ruled out. The most widely used methods for determining antioxidant activity are those that involve the generation of radical species, whereby the presence of antioxidants determines the disappearance of radicals (Cao *et al*., 1993). It is important to use different assays, instead of a single assay to assess and compare the antioxidant capacity of a sample of extract.

A combination of four methods applied in this study gave valuable information to evaluate the antioxidant activity of *P. bleo* and *P. grandifolia* and could be recommended for other similar investigations. For *P. bleo*, the ethyl acetate extract showed the highest reducing capacity measured by Folin-Ciocalteau method and the highest antioxidant activity in ß-carotene bleaching assay significantly ($p < 0.05$). In contrast, it is the hexane extract of *P. bleo* showed significantly ($p < 0.05$) the highest antioxidant activity when determined by the scavenging effect on DPPH radical and the reducing power assay.

For *P. grandifolia*, the reducing capacity, DPPH scavenging and the ß-carotene bleaching activities of the ethyl acetate extract was significantly the highest among the extracts ($p < 0.05$). The hexane extract of *P. grandifolia* possessed significantly ($p < 0.05$) highest reducing power capacity among the extracts.

Based on the results of the antioxidant assays, it is thus suggested that the antioxidant activities of hexane extracts in both *Pereskia spp.* were not solely contributed by phenolic compounds but other antioxidants in the extracts. Some of the antioxidant compounds appeared in the ethyl acetate extracts might also appear in the hexane extracts or *vice versa*.

The synergism among the antioxidants in the mixture made the antioxidant activity, not only dependent on the concentration of antioxidant, but also on the structure and interaction among the antioxidants (Sun and Ho, 2005). This might explain why the extracts of *P. bleo* (methanol, hexane and water extracts) and *P. grandifolia* (methanol and water extracts) with nearly similar reducing capacity measured by Folin-Ciocalteau method varied remarkably in their antioxidant activity.

4.3 Antimicrobial activity of *P. bleo* and *P. grandifolia* extracts

There are many methods to determine antimicrobial activities but there is no single all-embracing bioassay to evaluate the antimicrobial activity of a sample. Therefore, the evaluation process generally involves the use of a number of antimicrobial bioassay methods and careful comparison of all the data in order get an appropriate conclusion (Rahman *et al*., 2001). There are three major methods for antimicrobial testing: (i) agar diffusion method, (ii) broth dilution method and (iii) bioautographic method. For the current study, agar diffusion method and broth dilution method was chosen to determine the antimicrobial activity of the plant extracts.

In the agar diffusion method, paper disc impregnated with certain amount of tested sample are placed on the surface of the medium. The plates were incubated and

the zones of inhibition around each disc are measured. On the other hand, the broth dilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (Destoumieux *et al.*, 1999). The MIC was defined as the lowest concentration of the test sample at which the microorganism does not demonstrate visible growth, determined by inoculating the bacteria into a culture medium containing various concentrations of a proposed antibiotic. The MBC was determined as the lowest concentration at which no growth occurred on the agar plate (Randrianarivelo *et al*., 2009).

This study is a preliminary evaluation of antimicrobial activity of *P. bleo* and *P. grandifolia* extracts. The antimicrobial activity of *P. bleo* and *P. grandifolia* extracts were tested against two Gram positive bacteria (namely *Staphylococcus aureus* and *Bacillus subtilis*) and two Gram-negative bacteria (namely *Escherichia coli* and *Pseudomonas aeruginosa*). Generally, the positive controls (gentamycin and ampicilin) utilized to evaluate the efficacy of the extracts are standard antibiotics as indicated for each microorganism.

To our knowledge, little information is available on the antimicrobial properties of *P. bleo* and no information on *P. grandifolia*. Ruegg *et al.* (2006) reported that the methanol and dichloromethane extracts of the aerial part of *P. bleo* did not show active antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Mycobacterium smegmatis*, *Candida albicans*, *Pseudomonas aeruginosa* and *Helicobacter pylori*.

4.3.1 Agar disc diffusion assay of *P. bleo* and *P. grandifolia* extracts

Agar disc diffusion method of antimicrobial susceptibility test was developed in 1940s. In 1940, Heatley introduced the use of adsorbent paper for carrying antimicrobial solutions. The procedure, which is accepted by the National Committee for Clinical Laboratory Standards (NCCLS), is a modification of that described by Bauer, Kirby, Sherris and Turk (commonly known as the Kirby-Bauer test).

The results of the antimicrobial activity measured by zones of inhibition of the extracts and positive reference standards (gentamycin and ampicilin) are shown in Table 4.8. The antimicrobial effect of the positive reference standards (gentamycin and ampicilin) were relatively more pronounced compared to the tested extracts. However, there is no agreement on the level of acceptance for plant extracts when compared with standards. No zones of clearance were produced by the DMSO only control disc (negative control).

None of the extracts showed activity against *Escherichia coli*. The water extracts of both *Pereskia spp*. showed no inhibition against all the bacteria tested in this study. Generally, among the investigated extracts of each species, the ethyl acetate extracts exhibited the highest antibacterial effect.

For *P. bleo*, none of the extracts showed activity against *Escherichia coli* and *Staphylococcus aureus*. The methanol, hexane and ethyl acetate extracts of *P. bleo* at concentration of 500 mg/ml exhibited modest inhibition at 9.8, 9.5 and 8.5 mm respectively against *Pseudomonas aeruginosa*. When the concentrations of these three extracts are lowered to 50 mg/ml, a slight decline in the inhibition zone were shown by the methanol and ethyl acetate extracts whilst the hexane extract showed no inhibition at all (Table 4.8). The hexane and ethyl acetate extracts of *P. bleo* also showed modest

inhibition against *Bacillus subtilis* at 8.2 and 7.8 mm, respectively at 500 mg/ml concentration.

However, only the ethyl acetate extract of *P. grandifolia* showed some mild antimicrobial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis* at concentration of 500 mg/ml, with inhibition of 8.0, 9.2 and 8.5 mm respectively.

In summary, both *Pereskia spp.* did not demonstrate any promising antimicrobial activity against all tested bacterial in agar diffusion assay. The ethyl acetate extracts showed some mild activity among the extracts of each species.

Table 4.8: Results of the antimicrobial tests of the investigated plants in agar diffusion assay

Plant	Extracts	Concentration (mg/ml)	Inhibition zone (mm)[a] against			
			Gram negative		Gram positive	
			E. coli	*P. aeruginosa*	*S. aureus*	*B. subtilis*
P. bleo	Methanol	50	-	8.3 ±0.1	-	-
		500	-	9.8 ±0.1	-	-
	Hexane	50	-	-	-	-
		500	-	9.5 ±0.0	-	8.2 ±0.1
	Ethyl acetate	50	-	7.3 ±0.1	-	-
		500	-	8.5 ±0.1	-	7.8 ±0.0
	Water	50	-	-	-	-
		500	-	-	-	-
P. grandifolia	Methanol	50	-	-	-	-
		500	-	-	-	-
	Hexane	50	-	-	-	-
		500	-	-	-	-
	Ethyl acetate	50	-	-	-	-
		500	-	8.0 ±0.1	9.2 ±0.1	8.5 ±0.2
	Water	50	-	-	-	-
		500	-	-	-	-
Positive reference standards	Gentamycin (10 µg/disc)		20.7	18.0 ±0.1	22.0 ±0.5	19.0 ±0.0
	Ampicilin (10 µg/disc)		16.0	32.5 ±0.2	37.0 ±0.0	38.5 ±0.1

Values expressed are mean ± standard deviation of triplicate measurements.

-: no activity. Negative controls did not show any activity; [a] Inhibition zones including the diameter of the paper disc (6 mm).

4.3.2 Broth dilution assay

(i) MIC and MBC of *P. bleo* extracts

Extracts with MIC and MBC values < 10 mg/ml were considered relatively active. The MIC and MBC values of *P. bleo* extracts were all above 20 mg/ml, ranging from 30 to > 50 mg/ml (Table 4.9). In most cases, the MBC values of *P. bleo* extracts were equivalent to the MIC values (bactericidal effect), except for both hexane and ethyl acetate extracts against *Escherichia coli* and *Bacillus subtilis*, for which the MBC values were higher than MIC values (bacteriostatic effect). The higher MBC value than MIC value of the extract suggested that the extract inhibited growth of the test microorganisms while being bactericidal at higher concentration. The results here generally demonstrated higher antibacterial activity against gram positive bacteria strains.

All the extracts showed weak antimicrobial activity against *Pseudomonas aeruginosa*, with the same MIC and MBC values of > 50 mg/ml. The water extract demonstrated the weak antimicrobial activity against all the tested bacteria with the MIC and MBC values of > 50 mg/ml in all cases. It seems that the antimicrobial compounds could belong to the lipophilic group rather than to the hydrophilic. Methanol extract exhibited the same MIC and MBC values > 50 mg/ml against all the tested bacteria, except against *Bacillus subtilis* with MIC and MBC values of 40 mg/ml, which is the lowest MIC value among the tested bacteria. The hexane extract showed the lowest MIC values against *Escherichia coli* (MIC value 30 mg/ml), followed by *Bacillus subtilis* (MIC value 40 mg/ml). The MBC values for both inhibitions were the same (> 50 mg/ml), which were higher than the MIC values. For

ethyl acetate extract, the lowest MIC value was detected against *Bacillus subtilis* (30 mg/ml), followed by MIC value against *Staphylococcus aureus* (40 mg/ml).

In summary, all the extracts demonstrated weak antimicrobial activity against the tested microorganisms, except the hexane and ethyl acetate extracts which showed some mild activity against *Escherichia coli* and *Bacillus subtilis* respectively with MIC values of 30 mg/ml.

(ii) MIC and MBC of *P. grandifolia* extracts

Table 4.9 shows the MIC and MBC of *P. grandifolia* extracts after 24 h incubation with tested bacteria. All the extracts demonstrated weak antimicrobial activity against the tested microorganisms with MIC and MBC values of > 50 mg/ml, except ethyl acetate extract which showed very mild activity against *Bacillus subtilis* respectively with MIC values of 50 mg/ml.

Table 4.9: MIC and MBC of *P. bleo* and *P. grandifolia* extracts after 24 h incubation with bacteria

Plants	Extracts	*E. coli*		*P. aeruginosa*		*S. aureus*		*B. subtilis*	
		MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
P. bleo	Methanol	> 50	> 50	> 50	> 50	> 50	> 50	40	40
	Hexane	30	> 50	> 50	> 50	50	50	40	> 50
	Ethyl acetate	50	> 50	> 50	> 50	40	40	30	40
	Water	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50
P. grandifolia	Methanol	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50
	Hexane	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50
	Ethyl acetate	> 50	> 50	> 50	> 50	> 50	> 50	50	> 50
	Water	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50

4.3.3 Comparison of antimicrobial activity of *P. bleo* and *P. grandifolia*

Many synthetic antimicrobial agents have been introduced in clinical practices. However, in recent years, a number of antibiotics have lost their effectiveness due to the drug resistance and organ toxicity especially when these antibiotics are used for an extended period.

The antimicrobial activity of the tested extracts against gram negative bacteria was either low or inactive. Generally, gram negative bacteria i.e. *Escherichia coli* show higher resistance against the antimicrobial agents. *Escherichia coli* which was studied in this project, is one of the insensitive gram negative bacteria strains. In fact, gram negative bacteria, especially *Escherichia coli* are frequently reported to have multi drug resistance against many antibiotics available in the market (Alonso *et al*., 2000; Sader *et al*., 2002). As a result, it is not surprising that all the extracts tested in agar disc diffusion assay were not responding against *Escherichia coli*.

The results of the zone in the disc diffusion assay showed some correlation with the MIC and MBC obtained using a standardized broth dilution technique. From the antimicrobial assays in this project, the extracts of both *Pereskia spp.* were not demonstrated promising results against the tested microorganisms, which were *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis*. The results here were thus in agreement with Ruegg *et al.* (2006), which reported that the methanol and dichloromethane extracts of the aerial part of *P. bleo* were not showed active antimicrobial activity against *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*. However, the extracts of *Pereskia spp.* were only tested against four microorganisms in this project. Thus, it cannot be concluded that the *Pereskia spp.* are not active against all the microorganisms.

The ethyl acetate extracts of both *Pereskia spp.* showed some mild activity against the tested bacteria. There might be some active antimicrobial components in the extracts but low in quantity and were not present in sufficient concentrations to be effective. The active components of the extract may be characterized and tested for their safety and efficacy to uncover their therapeutic potential in modern and traditional medicine against infectious diseases. Alternatively, study of the synergistic interaction of active antimicrobial compounds isolates from the ethyl acetate extracts with commercial antibiotics might be useful to exploit these potential extracts in the combination therapy of infectious diseases caused by multi drug-resistant microorganisms.

4.4 *In vitro* neutral red cytotoxicity assay

Cytotoxicity assay is a rapid, standardized, sensitive and inexpensive method to measure drug-induced alterations in metabolic pathways or structural integrity which may or may not be related directly to cell death (Wilson, 1986). Cytotoxicity assays are widely used in *in vitro* toxicology studies.

The neutral red assay was chosen in this study to determine cytotoxic effect of *Pereskia spp.* extracts and isolated compounds against selected human cell lines, with doxorubicin as the positive control. The selected human cell lines were human nasopharyngeal epidermoid carcinoma cell line (KB), human cervical carcinoma cell line (CasKi), human colon carcinoma cell line (HCT 116), hormone-dependent breast carcinoma cell line (MCF7), human lung carcinoma cell line (A549) and non-cancer human fibroblast cell line (MRC-5).

Briefly, the neutral red cytotoxicity assay is a rapid, reliable, inexpensive, simple, sensitive and reproducible quantitative *in vitro* assay for the screening of potentially toxic agents. It is a colorimetric assay measuring the uptake of the neutral red, a supravital dye, by functional lysosomes after the cells were exposed to test compounds over a range of concentrations. Living cells take up the neutral red, which is concentrated within the lysosomes of the cells.

Neutral red (3-amino-*m*-dimethylamino-2-methyl-phenazine hydrochloride) has been used previously for identification of vital cells in cultures (DeRenzis and Schechtman, 1973). It is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion, accumulating intracellularly in lysosomes, where it binds with anionic sites in the lysosomal matrix. Alterations of the cell surface or the sensitive lysosomal membrane can lead to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics result in a decreased uptake and binding of neutral red dye. Based on the rationale that damaged and dead cells will lose their ability to retain neutral red dye, it is possible to distinguish between viable, damaged or dead cells using the neutral red cytotoxicity assay. Thus, this assay can be applied to determine the cytotoxicity effects of a series of extracts (Fotakis and Timbrell, 2006; Borenfreund and Puerner, 1985, 1986).

Different cytotoxicity assays might give different results depending on the test agent and the cytotoxicity used. Assays like MTT and LDH, which are dependent on enzymatic reactions, might be influenced by enzyme inhibitors like chloroquine (Weyermann *et al*., 2005). Fotakis and Timbrell (2006) reported that the neutral red assay and the MTT assay are the most sensitive cytotoxicity assay that show statistically significant difference between the treated cells and the controls, especially in detecting early toxicity. They also suggested that the neutral red assay is a useful tool

to detect lysosomal damage when used in conjunction with other tests in order to distinguish between cytotoxicity and organelle damage. Furthermore, Weyermann *et al*. (2005) pointed out that inexpensive assay like the neutral red uptake assay is sufficient when a more expensive test kits fails.

In the present study, the stock materials of the test extracts and compounds were dissolved in 100 % DMSO. Five and ten microlitre of a solution of each test extract or compound obtained by diluting the stock solution with appropriate quantity of DMSO 10 % was added to each well. The small amount of DMSO present in the wells (maximum 0.5 %) was proven not to affect the experiments. Houghton and Raman (1998) also reported that at concentrations below 3 % v/v, DMSO is usually not toxic to the cells.

For the determination of the effective concentration of inhibition of cell proliferation, cell lines were incubated with the extract in multiple concentrations. The amount of neutral red dye accumulated can be extracted from the lysosomes and quantitated spectrophotometrically by comparing with neutral red dye recovered from untreated control cell culture. The results are expressed as IC_{50} values which can be obtained from concentration-response curves. The IC_{50} values are established using different concentrations of each test agent tested on the cells. The IC_{50} value is the effective concentration (µg/ml or µM) of test extract or compound that cause 50 % inhibition or cell death (Borenfreund and Puerner, 1986, Chapuis *et al*., 1988, Liebsch and Spielmann, 1995).

According to the US NCI (United States National Cancer Institute) plant screening program, a plant extract is generally considered to have active cytotoxic effect if the IC_{50} value, following incubation between 48 to 72 h, is 20 µg/ml or less,

while it is 4 μg/ml or less for pure compounds (Lee and Houghton, 2005; Boik, 2001; Geran *et al*., 1972, 1997; Swanson and Pezzuto, 1990).

4.4.1 Cytotoxic activity of *P. bleo* and *P. grandifolia* extracts

(i) Human nasopharyngeal epidermoid carcinoma cell line (KB)

The *in vitro* growth inhibitions of KB cells by *P. bleo* and *P. grandifolia* extracts are shown in Figure 4.14 and Figure 4.15, respectively. The percentage of inhibition of KB cells increased with increased extract concentrations. For both *Pereskia spp.*, the ethyl acetate extracts showed the most effective cytotoxicity when tested at the highest concentration of 100 μg/ml, followed by methanol extracts and hexane extracts. The water extracts showed less than 50 % inhibition rate when tested at high concentrations of 100 μg/ml.

Table 4.10 summarise the cytotoxicity (IC_{50} values in μg/ml) of both *Pereskia spp.* extracts towards KB cells. For *P. bleo*, the ethyl acetate extract possessed the strongest cytotoxic effect with an IC_{50} value of 4.5 μg/ml against KB cells, followed by methanol and hexane extracts with IC_{50} values of 6.5 μg/ml and 28.0 μg/ml, respectively.

For *P. grandifolia*, the hexane extract possessed the strongest cytotoxicity with an IC_{50} value of 5.0 μg/ml towards KB carcinoma cells. This is followed by ethyl acetate and methanol extracts with IC_{50} values of 16.0 μg/ml and 34.0 μg/ml, respectively. However, the IC_{50} values of water fractions were above 100 μg/ml for both *Pereskia spp.*

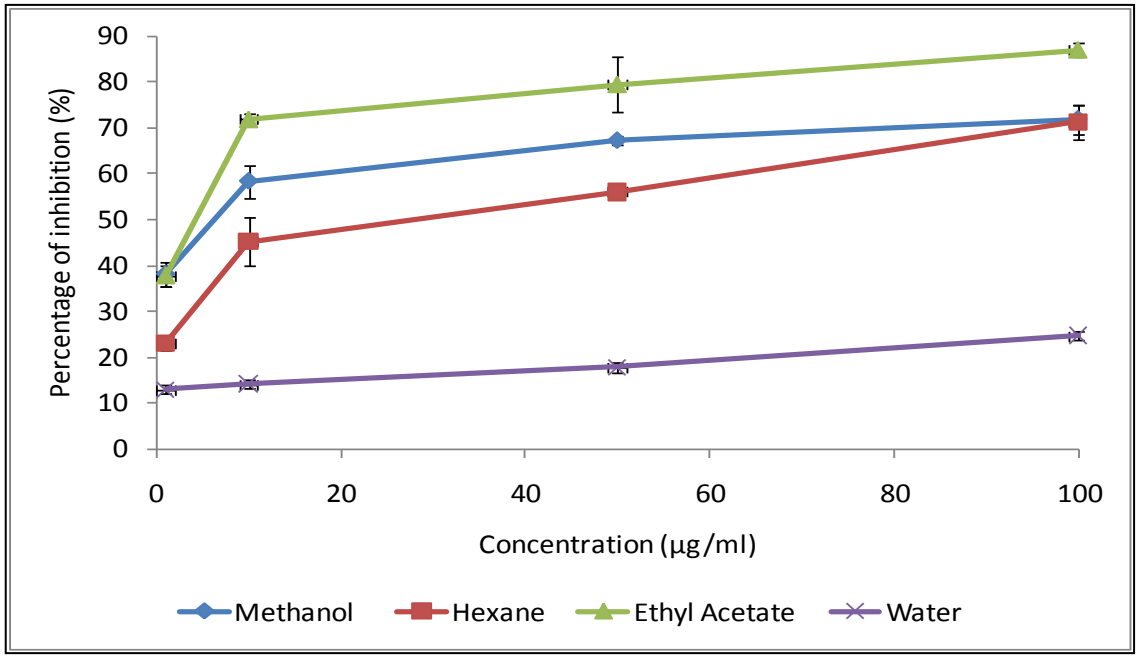


Figure 4.14: The *in vitro* growth inhibitions of KB cells by *P. bleo* extracts determined by neutral red cytotoxicity assay. Each value is expressed as mean ± standard deviation of three measurements.

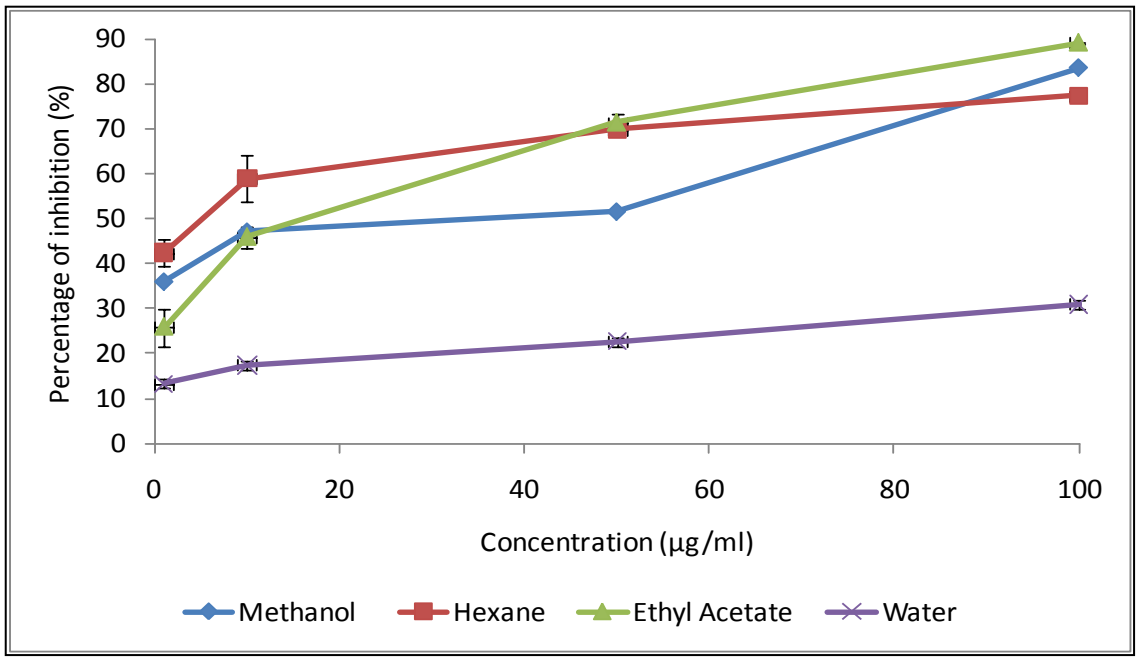


Figure 4.15: The *in vitro* growth inhibitions of KB cells by *P. grandifolia* extracts determined by neutral red cytotoxicity assay. Each value is expressed as mean ± standard deviation of three measurements.

Table 4.10: The IC_{50} values of *P. bleo* and *P. grandifolia* extracts tested against KB cell line

Plants	Extracts	IC_{50} values (μg/ml)
P. bleo	Methanol	6.5
	Hexane	28.0
	Ethyl Acetate	4.5
	Water	> 100.0
P. grandifolia	Methanol	34.0
	Hexane	5.0
	Ethyl Acetate	16.0
	Water	> 100.0

(ii) Human cervical carcinoma cell line (CasKi)

The ethyl acetate extracts of both *Pereskia spp.* showed the most effective cytotoxicity against CasKi cells when tested at the highest concentration of 100 μg/ml (Figure 4.16 and Figure 4.17) in comparison to other extracts. Water extracts of both *Pereskia spp*. showed very low inhibition rate at a high concentration of 100 μg/ml with the IC_{50} value of above 100 μg/ml.

Cytotoxicity (IC_{50} values in μg/ml) of *P. bleo* and *P. grandifolia* extracts towards CasKi cells are summarized in Table 4.11. For *P. bleo*, the crude methanol extract possessed the strongest cytotoxicity with an IC_{50} value of 40.5 μg/ml against CasKi carcinoma cells. This is followed by the ethyl acetate and hexane extracts with IC_{50} values of 58.0 μg/ml and 89.5 μg/ml, respectively. For *P. grandifolia*, the ethyl acetate extract possessed the strongest cytotoxicity with an IC_{50} value of 21.0 μg/ml towards CasKi carcinoma cells. This is followed by hexane and methanol extracts with IC_{50} values of 40.0 μg/ml and 50.0 μg/ml, respectively. It can thus be concluded that only the ethyl acetate extract of *P. grandifolia* has relatively active cytitixic effect on

the CasKi cell line based on the guideline proposed on cytotoxicity by the US NCI plant screening program.

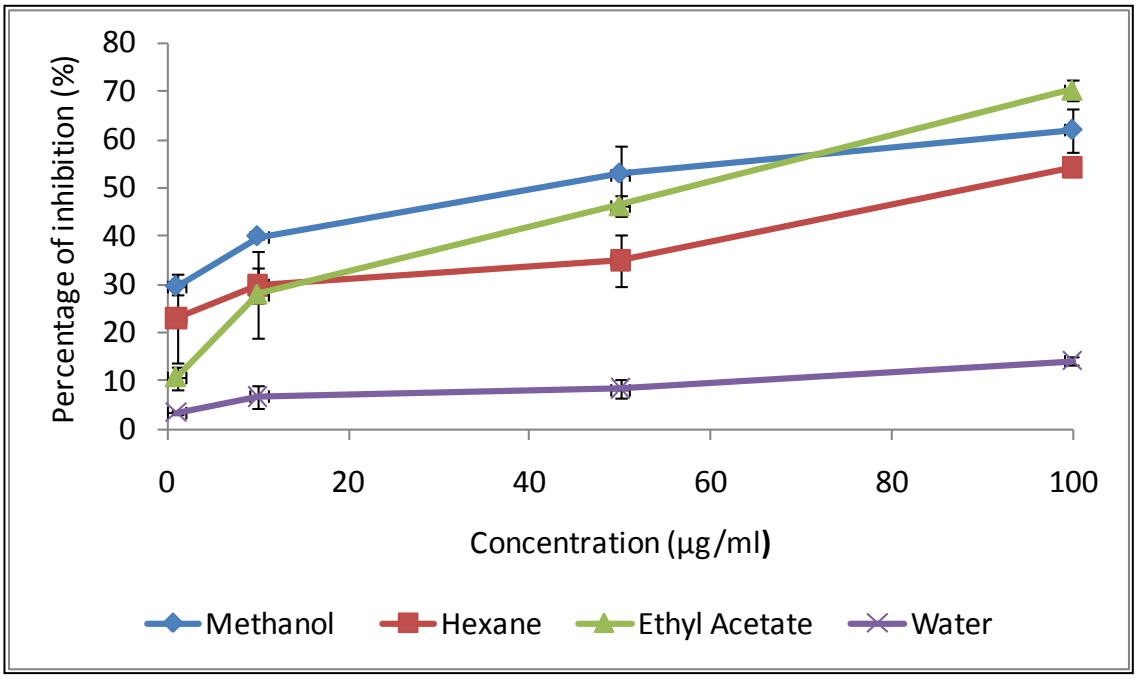


Figure 4.16: The *in vitro* growth inhibitions of CasKi cells by *P. bleo* extracts determined by neutral red cytotoxicity assay. Each value is expressed as mean ± standard deviation of three measurements.

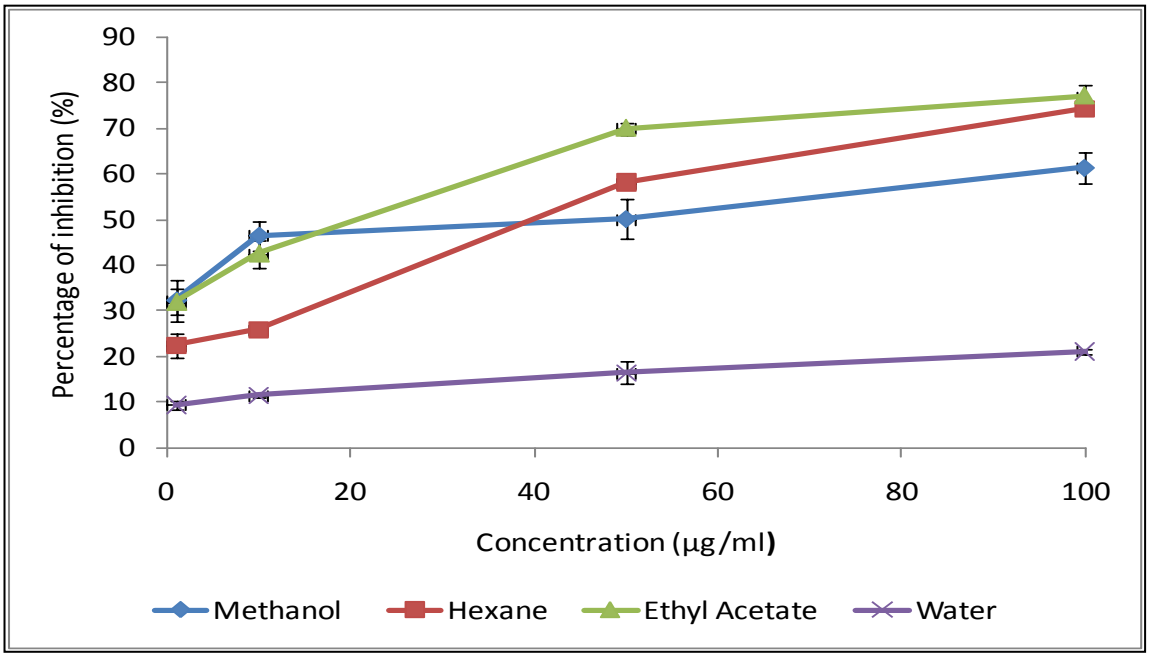


Figure 4.17: The *in vitro* growth inhibitions of CasKi cells by *P. grandifolia* extracts determined by neutral red cytotoxicity assay. Each value is expressed as mean ± standard deviation of three measurements.

Table 4.11: The IC_{50} values of *P. bleo* and *P. grandifolia* extracts tested against CasKi cell line

Plants	Extracts	IC_{50} values (μg/ml)
P. bleo	Methanol	40.5
	Hexane	89.5
	Ethyl Acetate	58.0
	Water	> 100.0
P. grandifolia	Methanol	50.0
	Hexane	40.0
	Ethyl Acetate	21.0
	Water	> 100.0

(iii) Human colon carcinoma cell line (HCT 116)

Cytotoxicity (IC_{50} values in μg/ml) of *P. bleo and P. grandifolia* extracts towards HCT 116 cells is summarized in Table 4.12. Percentage of inhibition of HCT 116 cells increased with increase extract concentrations. For *P. bleo*, the ethyl acetate extract possessed the strongest cytotoxicity with an IC_{50} value of 22.0 μg/ml towards HCT 116 carcinoma cells. This is followed by crude methanol and hexane extracts with IC_{50} values of 41.0 μg/ml and 67.5 μg/ml, respectively. However the IC_{50} value of water extract was above 100 μg/ml (Figure 4.18).

For *P. grandifolia*, the hexane extract possessed the strongest cytotoxicity with an IC_{50} value of 25.5 μg/ml towards HCT 116 carcinoma cells. This is followed by ethyl acetate and methanol extracts with IC_{50} values of 52.0 μg/ml and 53.0 μg/ml, respectively. However the IC_{50} value of water extract was above 100 μg/ml (Figure 4.19).

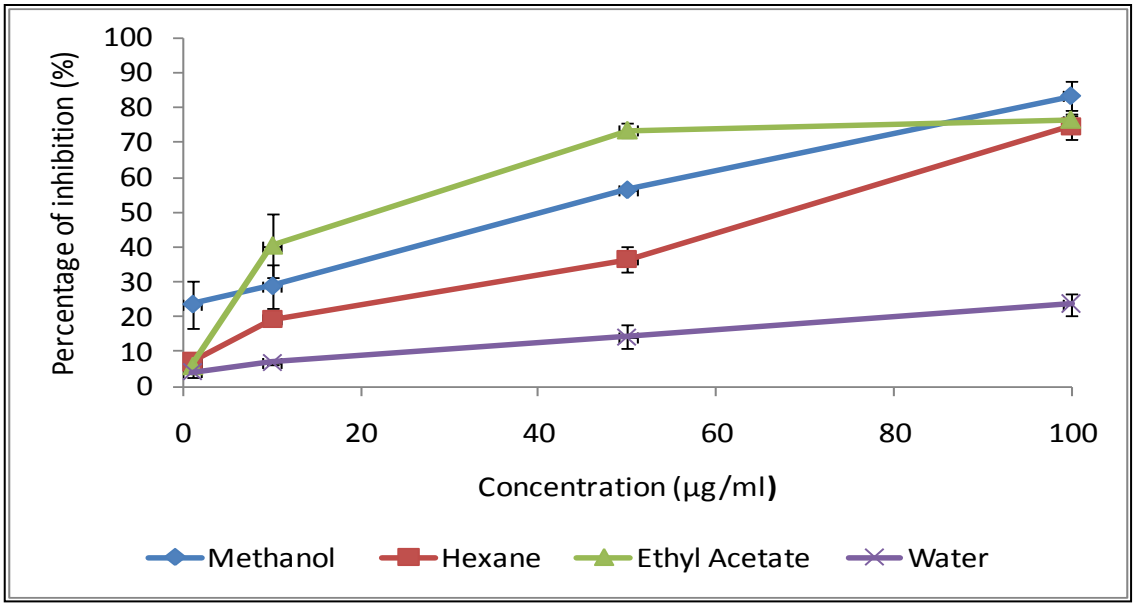


Figure 4.18: The *in vitro* growth inhibitions of HCT 116 cells by *P. bleo* extracts determined by neutral red cytotoxicity assay. Each value is expressed as mean ± standard deviation of three measurements.

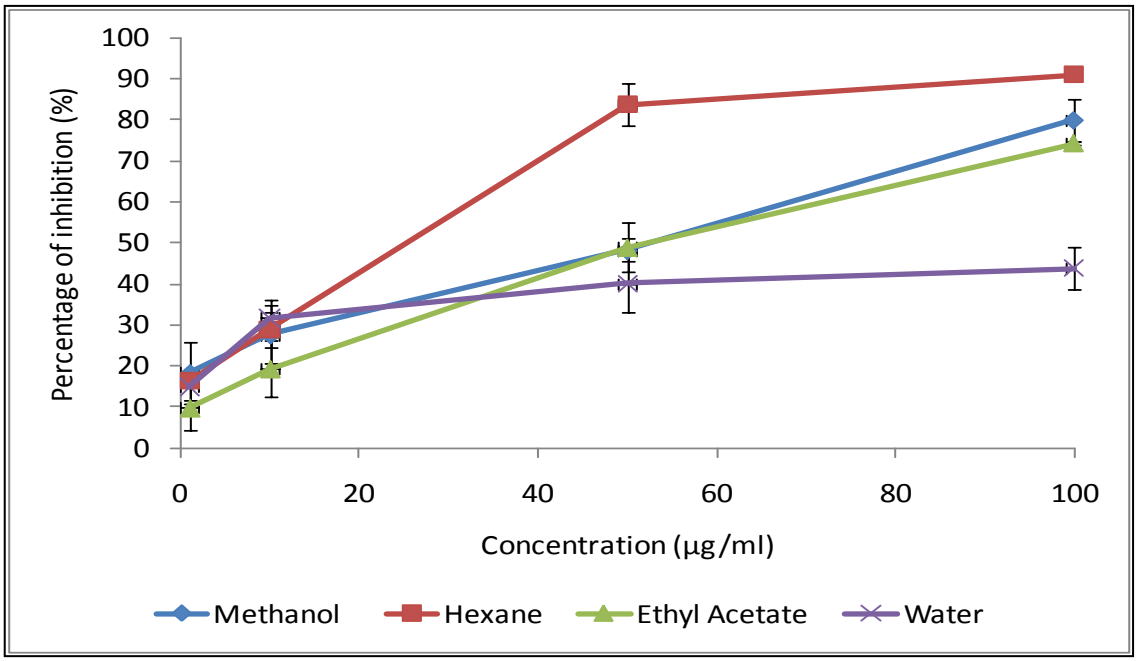


Figure 4.19: The *in vitro* growth inhibitions of HCT 116 cells by *P. grandifolia* extracts determined by neutral red cytotoxicity assay. Each value is expressed as mean ± standard deviation of three measurements.

Table 4.12: The IC_{50} values of *P. bleo* and *P. grandifolia* extracts tested against HCT 116 cell line

Plants	Extracts	IC_{50} values (μg/ml)
P. bleo	Methanol	41.0
	Hexane	67.5
	Ethyl Acetate	22.0
	Water	> 100.0
P. grandifolia	Methanol	53.0
	Hexane	25.5
	Ethyl Acetate	52.0
	Water	>100.0

(iv) Hormone-dependent breast carcinoma cell line (MCF7)

Cytotoxicity (IC_{50} values in μg/ml) of *P. bleo and P. grandifolia* extracts towards MCF7 cells is summarized in Table 4.13. Percentage of inhibition of MCF7 cells increased with the increase extract concentrations. For *P. bleo,* the hexane extract possessed the strongest cytotoxicity with an IC_{50} value of 25.0 μg/ml towards MCF7 carcinoma cells (Figure 4.20). This is followed by ethyl acetate and methanol extracts with IC_{50} values of 28.0 μg/ml and 39.0 μg/ml, respectively. However the IC_{50} value of water fraction was above 100 μg/ml.

For *P. grandifolia*, the ethyl acetate extract possessed the strongest cytotoxicity with an IC_{50} value of 20.0 μg/ml towards MCF7 carcinoma cells (Figure 4.21). This is followed by hexane and methanol extract with IC_{50} values of 49.0 μg/ml and 88.0 μg/ml, respectively. However the IC_{50} value of water fraction was above 100 μg/ml.

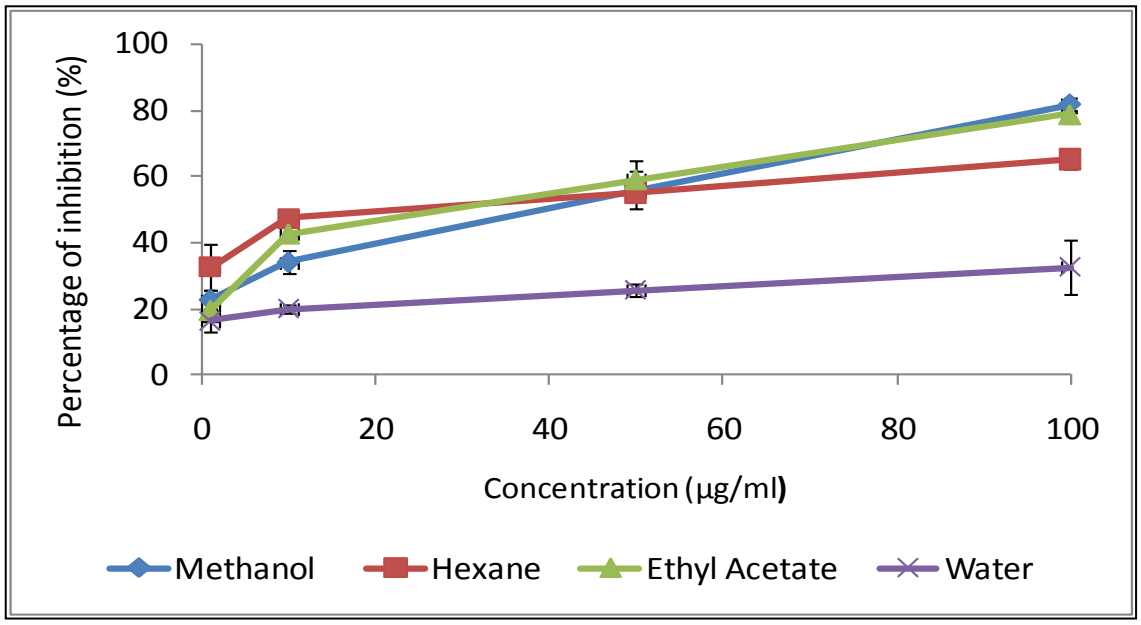


Figure 4.20: The *in vitro* growth inhibitions of MCF7 cells by *P. bleo* extracts determined by neutral red cytotoxicity assay. Each value is expressed as mean ± standard deviation of three measurements.

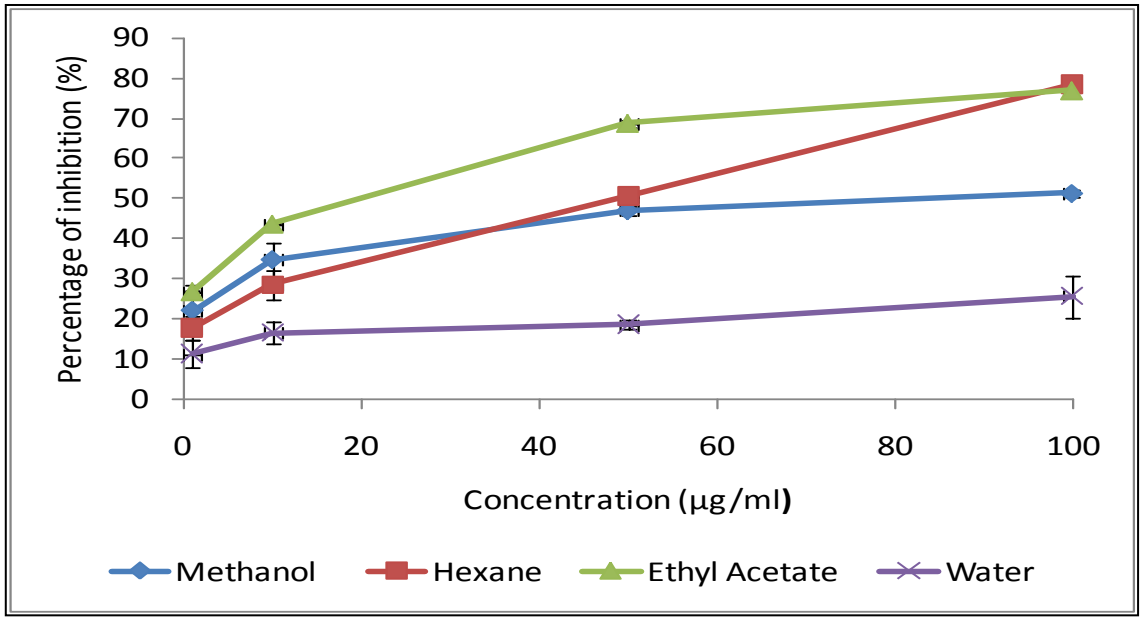


Figure 4.21: The *in vitro* growth inhibitions of MCF7 cells by *P. grandifolia* extracts determined by using neutral red cytotoxicity assay. Each value is expressed as mean ±standard deviation of three measurements.

Table 4.13: The IC_{50} values of *P. bleo* and *P. grandifolia* extracts tested against MCF7 cell line

Plants	Extracts	IC_{50} values (µg/ml)
P. bleo	Methanol	39.0
	Hexane	25.0
	Ethyl Acetate	28.0
	Water	> 100.0
P. grandifolia	Methanol	88.0
	Hexane	49.0
	Ethyl Acetate	20.0
	Water	> 100.0

(v) Lung carcinoma cell line (A549)

The *in vitro* growth inhibitions of A549 cells by *P. bleo* and *P. grandifolia* extracts are shown in Figure 4.22 and Figure 4.23, respectively. Percentage of inhibition of A549 cells increased with the increase extract concentrations. Cytotoxicity (IC_{50} values in µg/ml) of crude extract and fractions of *P. bleo* and *P. grandifolia* towards A549 cells are summarized in Table 4.14.

For *P. bleo*, the ethyl acetate extract possessed the strongest cytotoxicity with an IC_{50} value of 41.0 µg/ml towards A549 carcinoma cells. This is followed by methanol extract with IC_{50} value of 56.0 µg/ml. However the IC_{50} values of hexane and water extracts were above 100 µg/ml.

For *P. grandifolia*, the hexane, ethyl acetate and methanol extracts possessed cytotoxicity with IC_{50} values of 44.0, 45.0 and 60.0 µg/ml respectively towards A549 carcinoma cells. However the IC_{50} value of water extract was above 100 µg/ml.

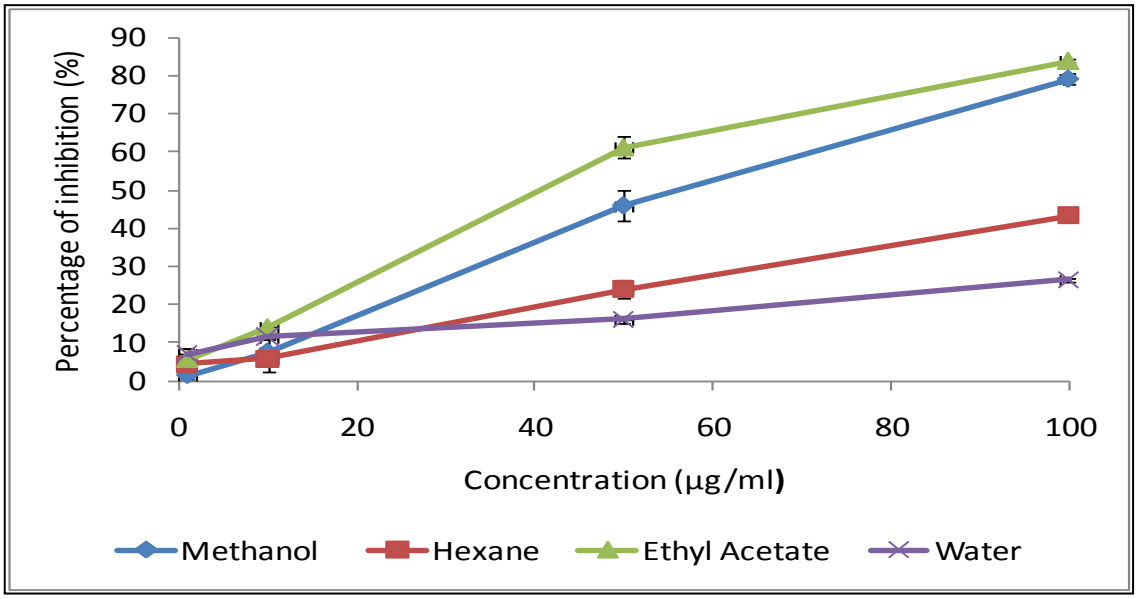


Figure 4.22: The *in vitro* growth inhibitions of A549 cells by *P. bleo* extracts determined by neutral red cytotoxicity assay. Each value is expressed as mean ± standard deviation of three measurements.

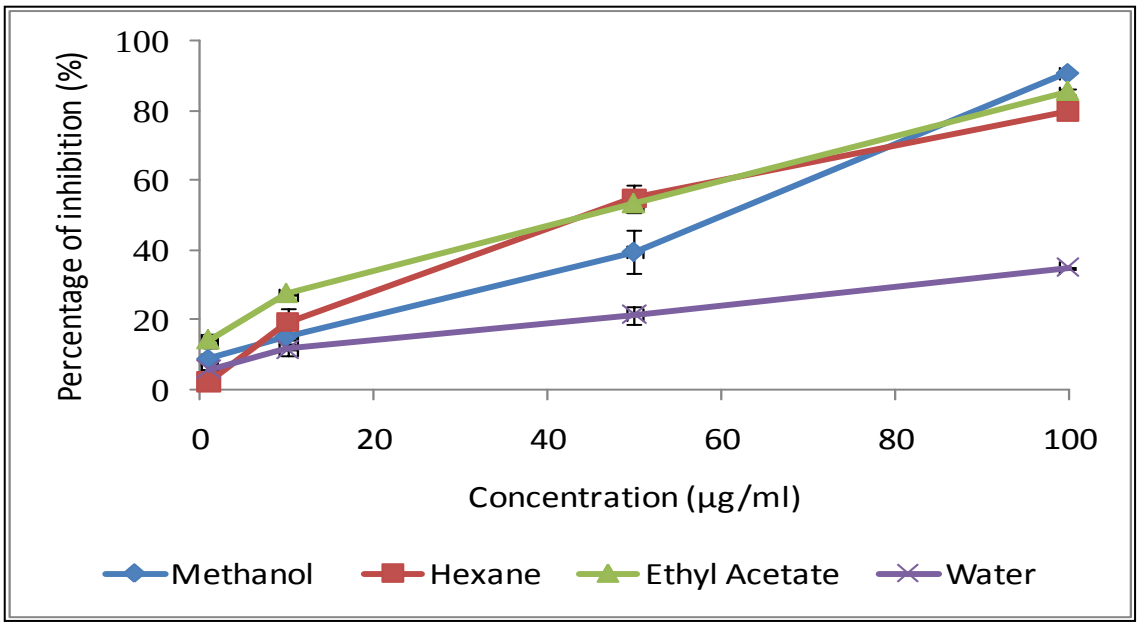


Figure 4.23: The *in vitro* growth inhibitions of A549 cells by *P. grandifolia* extracts determined by neutral red cytotoxicity assay. Each value is expressed as mean ± standard deviation of three measurements.

Table 4.14: The IC_{50} values of *P. bleo* and *P. grandifolia* extracts tested against A549 cell line

Plants	Extracts	IC_{50} values (µg/ml)
P. bleo	Methanol	56.0
	Hexane	> 100.0
	Ethyl Acetate	41.0
	Water	> 100.0
P. grandifolia	Methanol	60.0
	Hexane	44.0
	Ethyl Acetate	45.0
	Water	> 100.0

(vi) Human fibroblast cell line (MRC-5)

The IC_{50} values (µg/ml) of *P. bleo* and *P. grandifolia* towards MRC-5 cells is summarized in Table 4.15. For *P. bleo*, all extracts possessed low cytotoxic towards MRC-5 cells. The IC_{50} values of all fractions are above 100 µg/ml, except methanol extract with IC_{50} value of 61.0 µg/ml (Figure 4.24).

For *P. grandifolia*, all methanol extract and fractions of *P. grandifolia* had low cytotoxic activity towards MRC-5 cells. The IC_{50} values of all fractions are above 100 µg/ml, except methanol extract with IC_{50} value of 68.5 µg/ml (Figure 4.25).

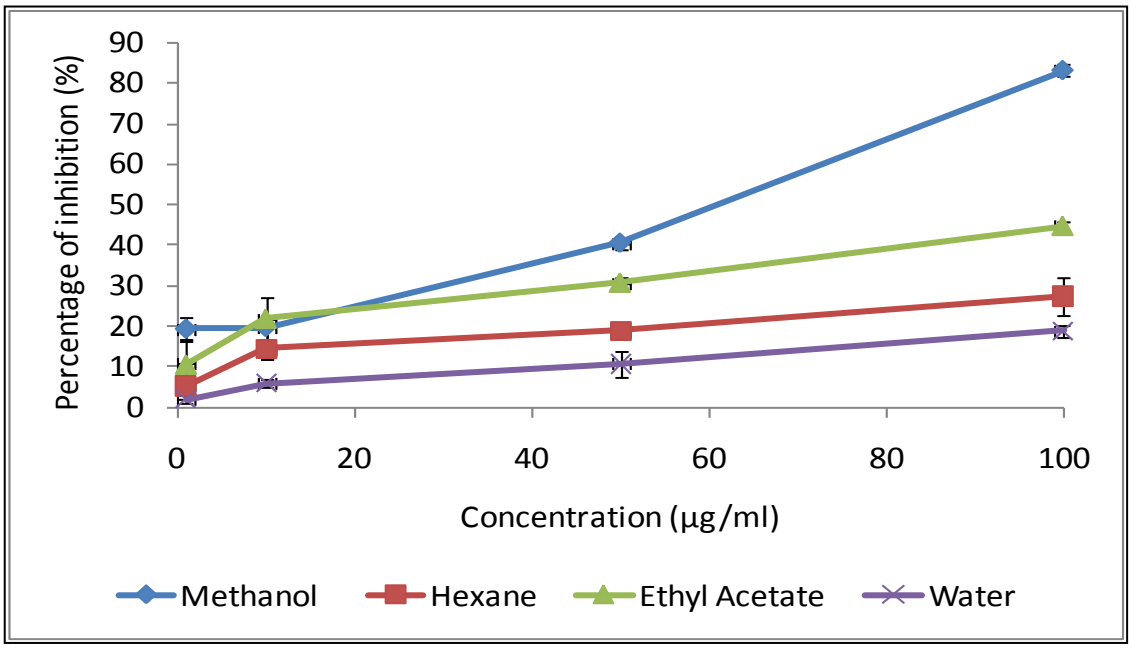


Figure 4.24: The *in vitro* growth inhibitions of MRC-5 cells by *P. bleo* extracts determined by neutral red cytotoxicity assay. Each value is expressed as mean ± standard deviation of three measurements.

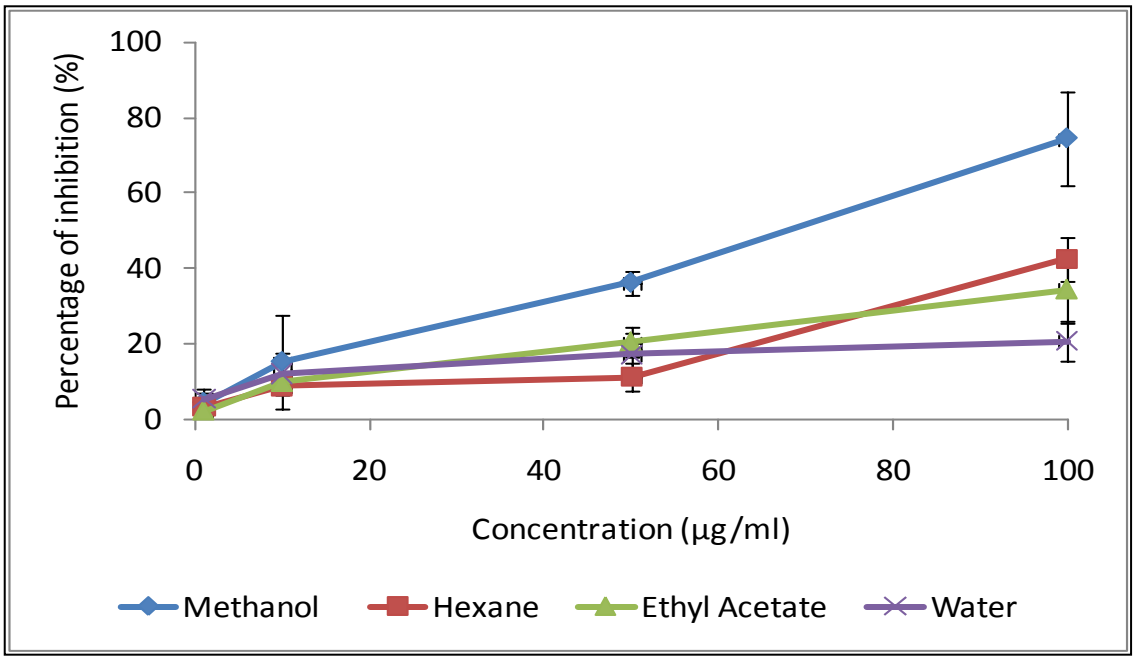


Figure 4.25: The *in vitro* growth inhibitions of MRC-5 cells by *P. grandifolia* extracts determined by neutral red cytotoxicity assay. Each value is expressed as mean ± standard deviation of three measurements.

Table 4.15: The IC_{50} values of *P. bleo* and *P. grandifolia* extracts tested against MRC-5 cell line

Plants	Extracts	IC_{50} values (µg/ml)
P. bleo	Methanol	61.0
	Hexane	> 100.0
	Ethyl Acetate	> 100.0
	Water	> 100.0
P. grandifolia	Methanol	68.5
	Hexane	> 100.0
	Ethyl Acetate	> 100.0
	Water	> 100.0

4.4.2 Cytotoxic activity of doxorubicin

Doxorubicin is clinically used for the treatment of a great variety of cancer disease (Gillle *et al*., 2002; Carter, 1975 and Khana *et al*., 1998). It has been one of the most extensively used agents in the chemotherapy regimens of cancer patients for the past 30 years (Weiss, 1992). In spite of the routine use of this drug its major adverse effect, the dose-dependent cardiotoxicity, cannot be prevented yet (Gillle *et al*., 2002). In the present study, doxorubicin was used as the positive control for the cytotoxicity assay. Thus, doxorubicin was screened for its cytotoxic activity on the selected human cancer cell lines, such as KB, CasKi, HCT 116, MCF7, A549 and non-cancer MRC-5 cell line.

Based on the result, it can be concluded that doxorubicin is not only cytotoxic against all the human cancer cell lines tested, but also the non-cancer human MRC-5 cell line (Table 4.16 and Figure 4.26). Doxorubicin possessed very strong cytotoxicity with IC_{50} values of 0.0125, 0.0060, 0.3600, 0.0755, 0.2200 and 0.5500 µg/ml against KB, CasKi, HCT 116, MCF7, A549 and MRC-5 cells respectively. This result supports

the statement that doxorubicin is a potent cytostatic drug which is applied for the treatment of cancer diseases but the routine use of this drug could bring major adverse effect (Gillle *et al*., 2002).

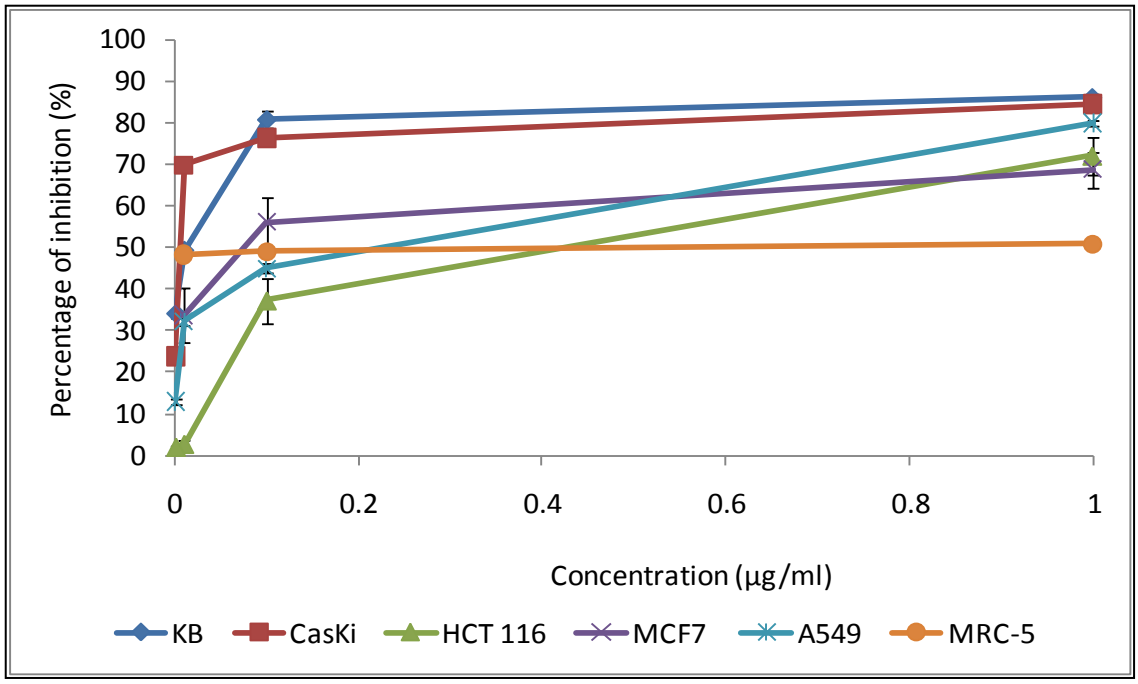


Figure 4.26: The *in vitro* growth inhibitions of selected human cells by doxorubicin as positive reference standard determined by neutral red cytotoxicity assay. Each value is expressed as mean ± standard deviation of three measurements.

Table 4.16: The IC_{50} values of doxorubicin against various cancer and non-cancer cell lines tested

Cell line	IC_{50} values in µg/ml (µM)
KB	0.0125 (0.023)
CasKi	0.0060 (0.011)
HCT 116	0.3600 (0.663)
MCF7	0.0755 (0.139)
A549	0.2200 (0.401)
MRC-5	0.5500 (1.01)

4.4.3 Comparison of cytotoxic activity of *P. bleo* and *P. grandifolia*

The results of preliminary cytotoxicity screening of the methanol and fractionated extracts of both *Pereskia spp.* against various cancer and non-cancer cell lines are summarized in Table 4.17.

The ethyl acetate extracts of both *Pereskia spp.* in general gave higher inhibition and stimulation values against various cancerous cell lines compared to other extracts. The ethyl acetate extract of *P. bleo* was selectively toxic against the KB cells. Both ethyl acetate extracts displayed distinctively cytotoxicity effect on KB cell line, which reached IC_{50} values at relatively low concentration. The ethyl acetate extract of *P. bleo* was more active with IC_{50} value of 4.5 μg/ml against KB cells, in comparison to 16.0 μg/ml shown by that of *P. grandifolia*. The ethyl acetate extract of *P. grandifolia*, on the other hand displayed good inhibition against MCF7 cells with IC_{50} of 20.0 μg/ml. The active ingredients in the ethyl acetate extract may lead to valuable compounds that may have the ability to kill KB cancer cells but exert no damage to normal cells (IC_{50} > 100.0 μg/ml against normal cells, MRC-5). The stronger inhibitory effect of the ethyl acetate extracts in comparison to the crude methanol extracts was probably due to the partial purification process whereby the active ingredients are concentrated in the ethyl acetate extract.

Meanwhile, the hexane extract of *P. grandifolia* also demonstrated remarkable high inhibition towards KB cells with IC_{50} value of 5 μg/ml, in comparison to the hexane extract of *P. bleo*. The water extracts of both *Pereskia spp.* were found to have no effect on the cancer cell lines (IC_{50} > 100.0 μg/ml in all cases). All the extracts of both *Pereskia spp.* demonstrated weaker cytotoxicity profile against the CasKi and

A549 cells compared to other cells and no activity against normal cells (IC_{50} > 100.0 μg/ml).

From Table 4.17, it is observed that the crude methanol extract of *P. bleo* showed a higher inhibitory effect than the fractionated extracts against the CasKi cells. This might be explained as the compounds that are active against the CasKi cells had decomposed during the fractionation process. In addition, observed activity in the total extracts might be due to synergism between components which were separated as a result of the fractionation process. The synergism among the components in the mixture contributed to the cytotoxic activity, not only dependent on the concentration of certain components, but also on the structure and interaction among the components.

In addition, the cytotoxic effect of the positive reference standards (doxorubicin) were relatively more pronounced than the tested extracts. Although the cytotoxic activities of these extracts are not as effective as doxorubicin, they however have low toxicity against normal MRC5 cell line in comparison to doxorubicin.

In summary, the findings from the cytotoxic activity of both *Pereskia spp.* extracts support the common belief that ethnopharmacological selection of *Pereskia spp.* is a useful criterion in drug discovery. It is interesting to note that the extract of the plant showed much less cytotoxicity against the normal cell line, and, if this also occurs *in vivo* (section 4.7), the use of this plant by locals for the treatment of cancer would have some scientific support. The active extracts were being investigated further to detect the DNA fragmentation (section 4.5) and to determine the active ingredients (section 4.8 and 4.9) present in the extract since these might identify valuable lead compounds in the light of their ability to kill cancer cells but exert little damage as possible on normal cells.

Table 4.17: Comparison between IC_{50} values of *P. bleo* and *P. grandifolia* extracts against various cancer and non-cancer cell lines

Plants / Standard	Extracts	IC_{50} (µg/ml)					
		KB	CasKi	HCT 116	MCF7	A549	MRC-5
P. bleo	Methanol	**6.5**	40.5	41.0	39.0	56	61.0
	Hexane	28.0	89.5	67.5	25.0	> 100	> 100.0
	Ethyl Acetate	**4.5**	58.0	22.0	28.0	41	> 100.0
	Water	> 100.0	> 100.0	> 100.0	> 100.0	> 100	> 100.0
P. grandifolia	Methanol	34.0	50.0	53.0	88.0	60	68.5
	Hexane	**5.0**	40.0	25.5	49.0	44	> 100.0
	Ethyl Acetate	**16.0**	21.0	52.0	**20.0**	45	> 100.0
	Water	> 100.0	> 100.0	> 100.0	> 100.0	> 100	> 100.0
Doxorubicin (Positive reference standard)		0.0125	0.0060	0.3600	0.0755	0.2200	0.5500

$IC_{50} \leq$ 20.0 µg/ml: Active

4.5 Detection of DNA fragmentation

Chromosomal DNA fragmentation at internucleosomal sites is the earliest hallmark of the nuclear events and the most extensively studied biochemical event in apoptosis. Apoptosis has become increasingly important to many areas of biomedical research and internucleosomal DNA fragmentation is frequently used to show the existence of apoptosis (Zhu and Wang, 1997).

In this study, DNA fragmentation was detected using DeadEnd™ Colorimetric Apoptosis Detection System as described by the manufacturer (Promega). DeadEnd™

Colorimetric Apoptosis Detection System is a modified TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling) assay, which actually takes advantage of the multiple free DNA ends generated by activated endonucleases to insert labeled dUTP that can be later detected by light microscopy. An analysis of DNA fragmentation with the DeadEnd™ Colorimetric Apoptosis Detection System can provide the first step toward the understanding of cell death mechanism.

4.5.1 DeadEnd™ Colorimetric Apoptosis Detection System (Promega)

The DeadEnd™ Colorimetric Apoptosis Detection System is a non-radioactive system designed to provide simple, accurate and rapid detection of apoptotic cells *in situ* at the single cell level. The system can be used to assay apoptotic cell death in cultured cells by measuring nuclear DNA fragmentation, an important biochemical indicator of apoptosis in many cell types.

Apoptotic cells have been clearly labeled in vibratome sections of rat brain after axotomy-induced neuronal death in the lateral geniculate nucleus (LGN) (Agarwala and Kalil, 1998; O'Brien *et al*., 1998), in Jurkat cells after anti-Fas treatment (O'Brien *et al*., 1998, Weis *et al*., 1992), and in HL-60 cells after anisomycin treatment (Polverino and Patterson, 1997). According to the technical bulletin of product (2005), the DeadEnd™ Colorimetric Apoptosis Detection System labels fragmented DNA *in situ* and have been tested in all of these systems.

The DeadEnd™ Colorimetric Apoptosis Detection System is ideal for labelling apoptotic nuclei in cultured cells, while allowing for a concurrent morphological assessment. The system end-labels the fragmented DNA *in situ* in apoptotic cultured cells. It is based on a modified TUNEL assay.

In the DeadEnd™ Colorimetric Apoptosis Detection System, biotinylated nucleotide is incorporated at the 3'-OH DNA ends using the Terminal Deoxynucleotidyl Transferase, Recombinat (rTdT) enzyme. Horseradish peroxidase-labeled streptavidin (Streptavidin HRP) is then bound to these biotinylated nucleotides, which are detected using the peroxidase substrate, hydrogen peroxide, and the stable chromogen, diaminobenzidine (DAB). Using this procedure, apoptotic nuclei are stained dark brown.

4.5.2 Induction of apoptosis by the cytotoxic active extracts of *P. bleo* and *P. grandifolia* on the selected human cancer cells

According to the results of preliminary cytotoxicity screening (section 4.4.3 and Table 4.17), the extracts of *P. bleo* (methanol and ethyl acetate) and *P. grandifolia* (hexane and ethyl acetate) were active against KB cells. The ethyl acetate extract of *P. grandifolia*, on the other hand, displayed a remarkable inhibition against MCF7 cells with IC_{50} of 20.0 μg/ml. In order to determine whether apoptosis play an important role in mediating cell death of the selected cells elicited by the active extracts of *P. bleo* and *P. grandifolia*, a modified TUNEL assay using DeadEnd™ Colorimetric Apoptosis Detection System (Promega) was carried out.

The cells treated with the cytotoxic active extracts produced dark brown stained nuclei with similar observation found in the positive control cells treated with DNase I (example: Figure 3.2) indicating the presence of DNA fragmentation which is the hallmark of apoptosis (Bowen *et al*., 1998). DNase I is an endonuclease that degrades both double-stranded and single-stranded DNA. None of the cell nucleus in the negative control cells were stained (Figure 3.2). Thus, it is tempting to speculate that the cell deaths elicited by the extracts were mediated *via* apoptotic mechanism.

It is well known that in apoptosis, the earliest recognized morphological changes are compaction and segregation of the nuclear chromatin, with the result of chromatin margination and condensation of the cytoplasm (Kerr *et al*., 1972). Progression of the condensation is accompanied by convolution of the nuclear and cell outlines followed by breaking up of the nucleus into discrete fragments and by budding of the cell as a whole to produce membrane-bounded apoptotic bodies. The apoptotic bodies are quickly ingested by nearby cells and degraded within their lysosomes (Kerr *et al*., 1972, 1994). The cellular events in apoptosis are accomplished quickly, with only a few min elapsing between onset of the process and the formation of cluster of apoptotic bodies. The KB cells treated with *P. bleo* (methanol and ethyl acetate extracts) and *P. grandifolia* (hexane and ethyl acetate extracts) and MCF7 cells treated with ethyl acetate extract of *P. grandifolia*, clearly demonstrated DNA fragmentation (Figure not shown), indicating apoptotic cell death as the major mechanism involved.

4.6 Determination of the expression level of apoptotic-related genes

Although a number of investigators have demonstrated a correlation between results obtained with the TUNEL method and ultrastructural features of apoptosis (Kimura *et al*., 1997; Li *et al*., 1995), others have reported that this method is not specific enough for apoptosis (Ansari *et al*., 1995; Stadelmann *et al*., 1998). Therefore, in addition to TUNEL which detected DNA fragmentation, gene expression level of apoptotic-related genes induced in the cells treated with active extracts of *Pereskia spp.* and isolated compounds, were evaluated to determine the mechanism of extract-induced apoptosis.

More recently, real-time florescent nucleic acid amplification techniques were developed and were proven to be rapid and sensitive (Chen *et al*., 2004). RT-qPCR significantly simplifies and accelerates the process of producing reproducible quantification of mRNAs (Bustin, 2000). However, this technique has not been commonly employed in natural products and there are no reports so far on LUX RT-qPCR methods for gene expression study in natural products.

In the present study, a protocol for the evaluation of mRNA expression levels of apoptotic genes by real-time reverse transcriptase–polymerase chain reaction (RT-qPCR) using LUX (Light Upon eXtension) primers has been established. The assay was started with preparation of quantitative standard (QSTD), followed by optimization of qPCR using LUX primers and application of optimized RT-qPCR assay to the experiment set (section 3.7). Only extracts or compounds which were active in the cytotoxic activity screening and detected with DNA fragmentation were tested in the gene expression study.

The QSTD of p53, caspase-3, c-myc and ß-actin were prepared by using one-step RT-PCR. These QSTD were used as DNA templates for optimization of RT-qPCR and also to prepare a standard curve for the RT-qPCR assay after 10-fold dilution. For relative quantification, the target PCR products were compared with these QSTD PCR products in which the concentrations were known. The aim of these QSTD was to provide a more accurate standard for quantification of nucleic acid. In addition, for effective quantification, the reaction must be stopped before amplification reaches the plateau phase (oversaturation) (Kellogg *et al*., 1990), so serial dilutions of the QSTD must be employed to ensure that the linear part of the curve is used.

4.6.1 The choice of using LUX chemistry for RT-qPCR assay in gene expression study

LUX primer technology, which has already been described for DNA and mRNA quantification approaches (Aitichou and Ibrahim, 2005; Chen *et al*., 2004) was used in the present study. The increase of the fluorescence signal is directly proportional to the amount of amplified DNA. The LUX primers combine the best of probe-based and SYBR Green detection. The LUX primer offers the sensitivity, specificity and dynamic range of TaqMan® probes, while providing the ease-of-use, cost-effectiveness and melting curve analysis capability of SYBR Green (Table 4.18). Unlike the current well known TaqMan® probes, LUX primers technology does not require an expensive probe (Chen *et al*., 2004). The LUX assay only needs a specific primer set with a single labeled, self-quenched primer and a corresponding unlabeled one. It is more reliable than the real-time method using DNA binding dyes (e.g. SYBR green), which is frequently used in qPCR reactions to monitor DNA synthesis. However, the disadvantage is that SYBR Green will bind to any dsDNA in the reaction, including primer-dimers and other non-specific reaction products, which results in an overestimation of the target concentration and may produce potentially misleading results due to the lack of specificity of the dyes. A previous study also indicated that the LUX primers technology is reliable for quantitation of gene expression and the result is similar to the probe-based quantitative assay (Brian *et al*., 2003). LUX fluorogenic primers can be designed and ordered via online software (http://www.invitrogen.com/lux).

Table 4.18: Comparison of chemistry options for real-time amplification

	TaqMan® Probes	Molecular Beacons	SYBR Green	LUX Primers
Sensitivity	•••	•••	•	•••
Dynamic range	•••	•••	•	•••
Specificity	•••	•••	•	•••
Multiplexing	••	••	N/A	•••
Melting curve analysis	N/A	N/A	•••	•••
Ease of design	•	•	•••	•••
Cost	•	•	•••	•••

• Sensitivity; N/A: Not available

The LUX assay also has the advantage of speed increment and is less laborious over the gel-based RT-PCR technique which is the popular gene expression tool applied in natural products. The LUX assay took 88 min to complete the amplification reaction (including the melting curve analysis) and the process was viewed in real time, while conventional RT-PCR methods usually take more about 3 hs or more for gene amplification (no melting curve analysis) and half an h or more to run the gel and examine the result. Furthermore, the LUX assay is a closed-tube technique which reduces the risk of contamination and reaction variability. Thus, the advantage of the LUX real-time assay is more apparent when compared to conventional method to measure gene expression.

4.6.2 Validation and optimization of the LUX RT-qPCR assay

The RT-qPCR is a complex assay and all physical and chemical components of the reaction are interdependent. Assay optimization is the key to better sensitivity, specificity, reproducibility and a wider linear dynamic range. Although optimization of PCR reactions used to be an essential part of assay development, recent trends towards

high throughput and rapid data reporting have resulted in the elimination of this step in many laboratories. Indeed, some manufacturers claim it is not necessary to optimize the assay when using their particular reagents or chemicals. However, it is clear that the optimization step can significantly improve assay detection sensitivity (Nolan *et al*., 2006).

In the present study, the PCR method used for preparation of QSTD was adapted to real time format by using LUX primers (Invitrogen), Platinum *Taq* DNA polymerase (Invitrogen) and dNTP mix (Promega), performed in Rotor-Gene 6000 (Corbette). For the optimization of the qPCR assay, an aliquot of the QSTD dilution was used as template for the qPCR amplification.

The optimization of a qPCR assay basically depends upon five factors: specificity test of primer, primer concentration, $MgCl_2$ concentration, annealing temperature and sensitivity of the test. The optimized qPCR assay should fulfil the following criteria in the given order of priority: (i) absence of, or minimal, primer dimmers; (ii) lowest Ct, i.e. the primer and $MgCl_2$ combination resulting in the most sensitive and efficient reaction; (iii) highest end point fluorescence (ΔRn), which is likely to signify the highest number of amplicon products being formed, and (iv) absence of positive signal in NTC (Nolan *et al*., 2006).

(i) Specificity test of LUX primers

Primers are the most crucial component in PCR reactions because they determine the sensitivity, specificity and efficiency of the assay. However, a primer might sometimes forms undesired secondary structural pairing such as primer dimmer artifacts. Primer dimer artifacts refer to the primers which anneal to themselves and

create small templates for PCR amplification. The presence of primer dimmers in samples containing template decreases the efficiency of a PCR reaction.

The sequences of the LUX primers (Table 3.8) used were designed by proprietary software called D-LUX Designer (Invitrogen, http://www.invitrogen.com/lux). The characteristics of the LUX primers, such as amplicon size, primer length and primer Tm, are included in the primer design by the software to output primer pairs that are located throughout the target (input) sequence. The software also performed a BLAST search of the designed sequence against known database to eliminate cross-reactivity.

Each fluorogenic LUX primer was labelled with one of two reporter dyes, either FAM (6-carboxy-fluorescein) or JOE (6-carboxy-4',5'-dichloro-2',7'-dimethoxy-flurescein). In the present study, FAM-labelled primer set was used to detect apoptotic related genes (p53, caspase-3 and c-myc) while JOE-labelled primer set was used to detect internal reference gene (ß-actin).

During the amplification, the real-time qPCR machine (Rotor-Gene 6000, Corbette) detected fluorescence in each cycle, excited and detected the FAM-labelled LUX primers at their excitation/emission wavelength of 470/510 nm in green channel while JOE-labelled LUX primers were excited and detected at 530/555 nm in yellow channel.

The specificity of each LUX primers (p53, caspase-3, c-myc and ß-actin) was assessed against the QSTD of each gene (p53, caspase-3, c-myc and ß-actin), to confirm that each set of primers amplified only the specific genome. The results are shown in Figure 4.27 and 4.28. Specific PCR products were further analyzed by melting curve analysis and the results are shown in Figure 4.29 and 4.30.

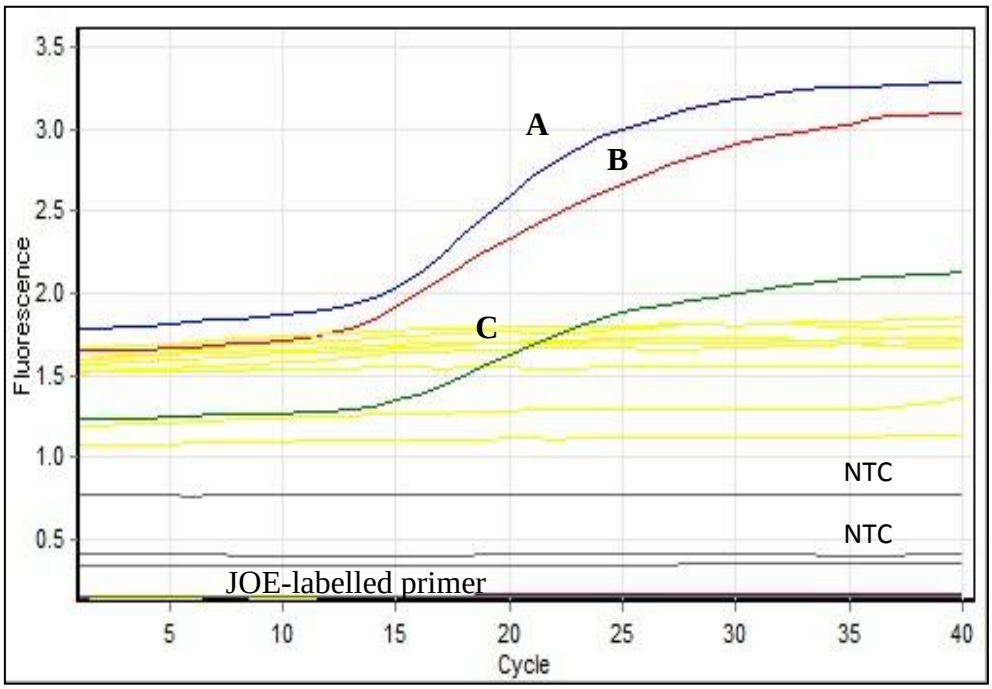


Figure 4.27: The specificity of FAM-labelled LUX primers in green channel. The specificity of four set of primers (c-myc, p53, caspase-3 and ß-actin) was established using the QSTD of the four apoptotic genes (c-myc, p53, caspase-3 and ß-actin). A: LUX primer of p53 against QSTD of p53; B: LUX primer of c-myc against QSTD of c-myc; C: LUX primer of p53 against QSTD of p53; NTC: non-template control; Yellow lines: non-specific genome of (c-myc, p53, caspase-3) of each LUX primers set.

As shown in Figure 4.27, the FAM-labelled primers (p53, caspase-3, c-myc) were correctly amplified the specific genome of each primer. Positive amplifications of each pair of LUX primer and QSTD generated sigmoid amplification curves. There was no amplification (flat line) detected for the non-specific genome of each LUX primers set (yellow flat lines in Figure 4.27) and NTC. The amplification curve of JOE-labelled primer (ß-actin LUX primer) was not shown in Figure 4.27 as JOE-labelled primer was not detected at wavelength of 470/510 nm in green channel. The JOE-labelled primer can only be detected at 530/555 nm in yellow channel (Figure 4.28). As shown in Figure 4.28, the ß-actin LUX primer, which was a JOE-labelled LUX primer was correctly amplified the genome of ß-actin.

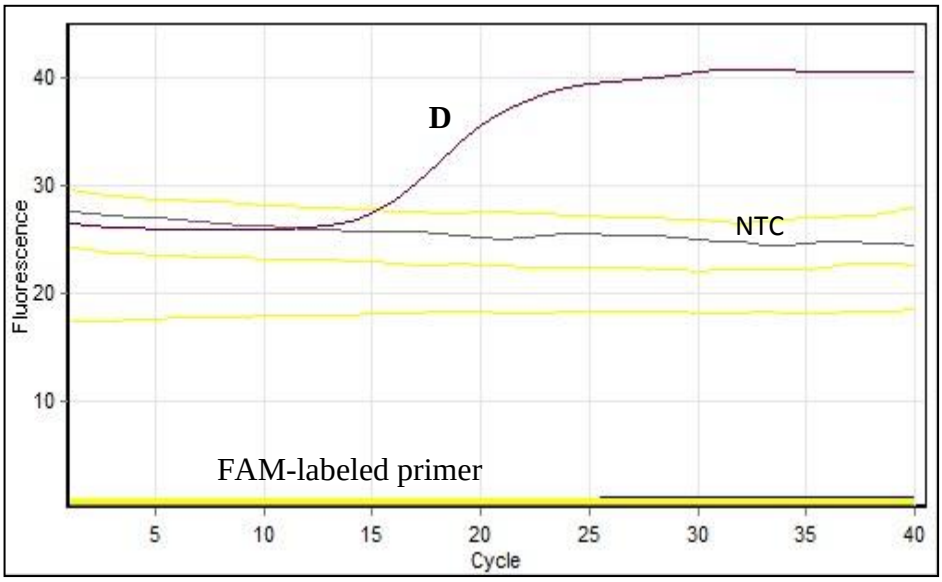


Figure 4.28: The specificity of JOE-labelled LUX primers in yellow channel. D: LUX primer of ß-actin against QSTD of ß-actin; NTC: non-template control; Yellow lines: non-specific genome (c-myc, p53, caspase-3) against ß-actin LUX primer.

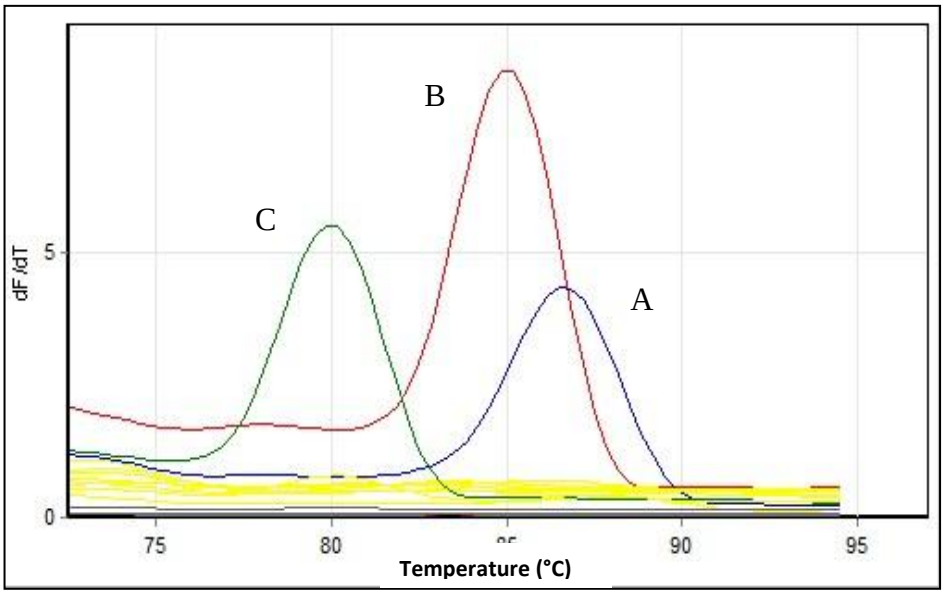


Figure 4.29: Melting curve of FAM-labelled LUX primers in green channel. A: LUX primer of p53 against QSTD of p53; B: LUX primer of c-myc against QSTD of c-myc; C: LUX primer of p53 against QSTD of p53; grey lines: NTC; Yellow lines: non-specific genome of (c-myc, p53, caspase-3) of each LUX primers set.

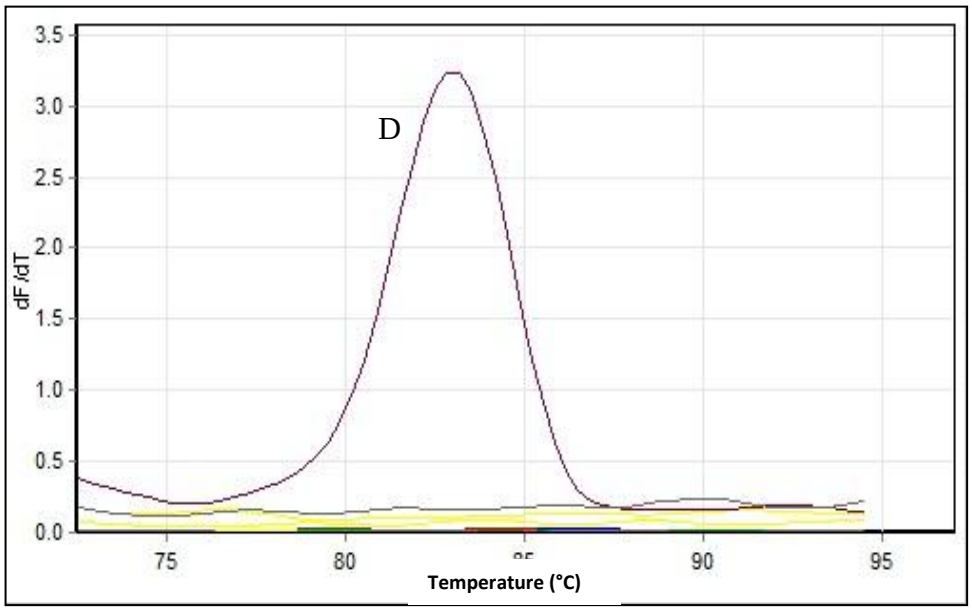


Figure 4.30: Melting curve of JOE-labelled LUX primers in yellow channel. D: LUX primer of ß-actin against QSTD of ß-actin; Grey line: non-template control; Yellow lines: non-specific genome (c-myc, p53, caspase-3) against ß-actin LUX primer.

Melting curve analysis during qPCR is important to identify the presence of primer dimmers, analyze the specificity of the reaction and confirm specific amplification with LUX primers. Specific PCR product melts in a specific manner produce a melting temperature (Tm) peak in melting curve analysis. There was no primer dimmers detected in the melting analysis of Figure 4.29 and 4.30, indicating that the designed LUX primers were specific and sensitive. In Figure 4.29 and 4.30, Tm for c-myc, p53, caspase-3 and ß-actin were 85 ℃, 86.5 ℃, 80 ℃ and 83 ℃, respectively. The difference between experimental Tm and Tm predicted by the software (Table 3.8) was due to the higher concentration of Mg^{2+} added in the experiment which increased the Tm of PCR products.

(ii) Optimization of the concentrations of LUX primers and $MgCl_2$

The thermodynamic stability (ΔG) of a primer differs for different primers and varies with primer concentration (Nolan *et al*., 2006). Therefore, it is important to use primers at concentrations that result in optimal hybridization and priming. Indeed, the manufacturers claim that it is not necessary to optimize primer concentrations when using their particular products. However, it is clear that the rationale underlying the original recommendations remain valid and that optimization of primer concentration can significantly improve assay detection sensitivity (Nolan *et al*., 2006).

In addition, Mg^{2+} concentrations required strict control because Mg^{2+} affects enzyme activity and increases the Tm of dsDNA (Eckert and Kunkel, 1991). The results indicated that the lowest detection limit for LUX primers and $MgCl_2$ were 0.3 μM and 3.0 mM respectively as both of the concentrations fulfilled the following criteria in the given order of priority: (i) absence of primer dimmers, (ii) lowest Ct, i.e. the primer and $MgCl_2$ combination resulting in the most sensitive and efficient reaction, (iii) absence of signal in NTC (Nolan *et al*., 2006).

(iii) Optimization of the annealing temperature (T_A)

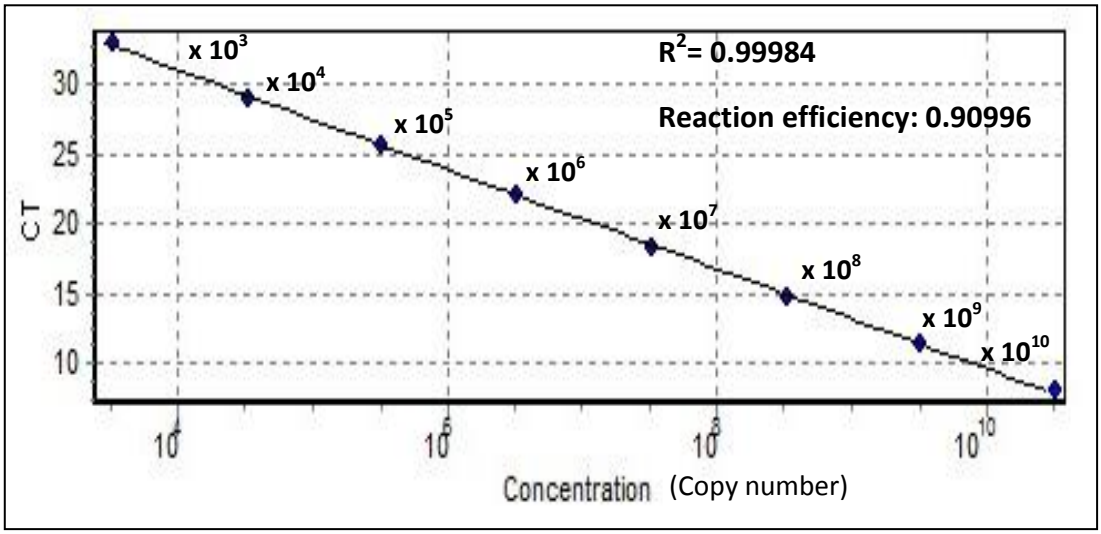
The setting of the PCR thermocycling profile would affect the overall performance of the PCR assay. The T_A is one of the key factors in determining the annealing rate of a specific primer. The Tm of a primer can be predicted by bioinformatics software though the actual T_A must be determined empirically based on the predicted Tm by performing gradient T_A PCR.

In the present study, three different T_A were tested for optimization, which were 50 ℃, 55 ℃ and 60 ℃. There was no significant different among these three T_A (data not shown). Thus, the T_A was set to 55 ℃ as suggested by the kit.

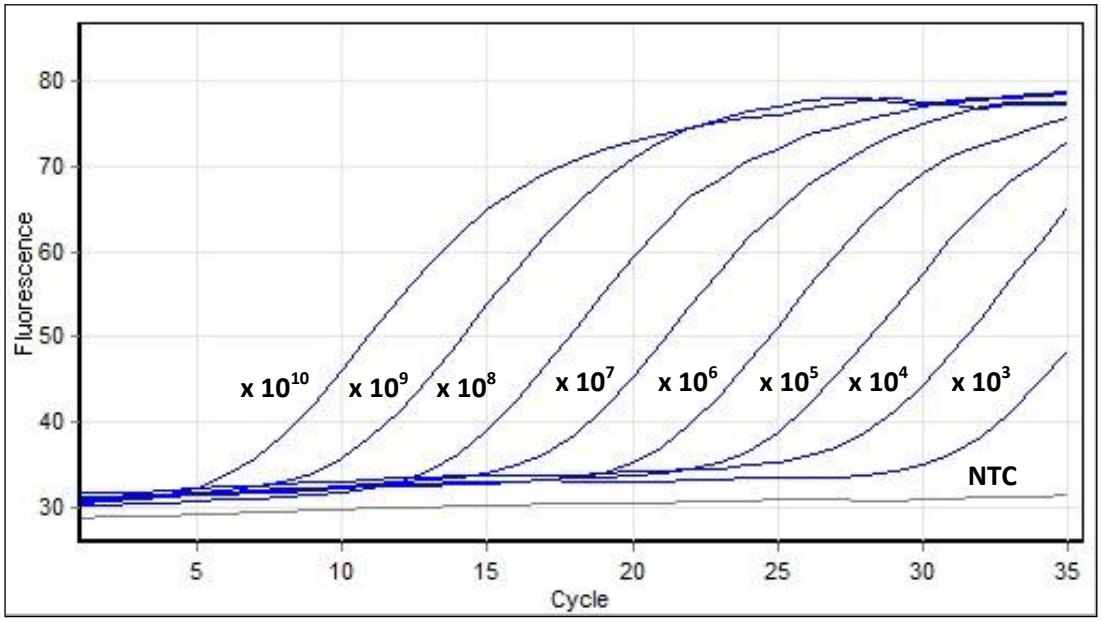
(iv) Determination of the lowest detection limit and sensitivity of the LUX RT-qPCR assay

According to previous report (Chen *et al*., 2004), the LUX assay was 10-fold more sensitive than the conventional RT-PCR method, while the amount of RNA used in the LUX method is only one-fifth of the gel based. In the present study, the lowest detection limit (or primer's sensitivity) of the LUX RT-qPCR were determined by performing amplification of 10-fold serial dilution of purified QSTD of each tested gene using LUX primers. A 10-fold difference in concentration corresponds to 3.3 cycles. For standard curve analysis, it is important to confirm that the slope of curve lies between -3.2 and -3.6, the R^2 value is above 0.98 and the reaction efficiency is above 0.90. The standard curve was prepared in every run of qPCR.

The lowest detection limits of the four QSTD genes were same, which was 10^3 copies while the reaction efficiencies of the four QSTD genes were > 0.90 and R^2 (coefficients of linear regression) of the dynamic range were > 0.99900 (example: Figure 4.31). The reaction efficiencies of p53, caspase-3, c-myc and ß-actin obtained were 0.99943. 0.99976, 0.99955 and 0.99984, respectively, while the R^2 of p53, caspase-3, c-myc and ß-actin obtained were 0.90673, 0.91393, 0.90304 and 0.90996, respectively. This indicates that the assay were linear over each of their quantitation range. Therefore, the lowest limit of quantitation of LUX assay was set at $x10^3$ copies/µl.



Standard curve



Amplification curve

Figure 4.31: The standard curve and amplification curve obtained by the LUX qPCR assays on the dilution series of ß-actin QSTD template. The lowest detection limit of ß-actin QSTD was 10^3 copies/µl.

4.6.3 Considerations for RT-qPCR procedures

(i) Reagents

The amplification depends on the activity and the quality of the enzyme *Taq* polymerase, availability of essential components such as dNTPs and the nature of the target DNA. Thus, the use of good PCR reagents or master mixes would have a strong effect on PCR efficiency. Oligo-dT was used during cDNA synthesis in the present study. It is more specific than random priming, and is the best method to use when the aim is to obtain a faithful cDNA representation of the mRNA pool. It is also the most appropriate choice when aiming to amplify several target mRNAs from a limited RNA sample (Bustin *et al.*, 2005).

(ii) Negative controls

Every run of qPCR assay of each extract (or compound tested) for each gene, was included cDNA of samples, QSTD dilutions, non-reverse-transcriptase control (NRTC) and non-template control (NTC). The QSTD dilutions, NRTC and NTC were used during the entire assay development and assay evaluation.

Amplification of QSTD standard curve was included, analyzed and data reported to reveal the amplification efficiency and sensitivity of every PCR assay, to check for consistency of reaction and to confirm that the quantification of samples (at 6 time points) has been carried out within the dynamic range of that assay.

It is virtually impossible to completely eliminate genomic DNA from RNA preparations. Therefore, it is important to include negative controls in RT-qPCR experiments. Two types of negative controls were used in the present study, which were NRTC and NTC. NRTC was a minus reverse-transcriptase control (or "no

amplification control") that containing the master mix reagents and the RNA template without reverse transcriptase procedure. If a product was seen in the NRTC, it probably indicated that contaminating DNA was present in the sample.

On the other hand, NTC included all of the RT-PCR reagents except the DNA/RNA template. Typically the RNA/DNA template was simply substituted with nuclease-free water. NTC was essential to check for non-specific signal arising from primer dimmers or template contamination. No product should be synthesized in the NTC; if a product was amplified, it indicated that one or more of the RT-PCR reagents was contaminated with the amplicon.

(iii) Internal reference gene

The reliability of any RT-qPCR experiment can be improved by including an invariant internal reference gene (or so-called endogenous reference or housekeeping genes) in the assay to correct for sample to sample variations in RT-qPCR efficiency and errors in sample quantification. The expression level of a good control should not vary across the samples being analyzed.

The ideal internal standard should be expressed at a constant level among different tissues of an organism, at all stages of development, and should be unaffected by the experimental treatment. In addition, an internal reference gene should also be expressed at roughly the same level as the RNA under study (Bustin, 2000). There are three RNAs which are most commonly used to normalize patterns of gene expression, which are glyceraldehydes-3-phosphate-dehydrogenase (GADPH), ß-actin and ribosomal RNAs (rRNA).

GADPH is particularly an unpopular choice in cancer research due to its increased expression in aggressive cancers (Goidin, 2001). The issue of the choice of an internal control has also been reviewed by Suzuki *et al.* (2000). The authors recommended caution in the use of GADPH as a internal control as it has been shown that its expression may be regulated in proliferating cells and recommended ß-actin as a better active reference. GADPH is severely criticized as internal control by others too (Bustin, 2000; Dheda, 2004; Aerts *et al*., 2004).

In the present study, the mRNA level of ß-actin was used as an internal control for template levels. ß-actin mRNA is expressed at moderately abundant levels in most cell types and encodes a ubiquitous cytoskeleton protein. It was one of the first RNAs to be used as an internal standard, and it is still advocated as a quantitative reference for RT-PCR assays (Kreuzer *et al*., 1999). This is despite widespread evidence that its levels of transcription can vary widely in response to experimental manipulation in human breast epithelial cells, and blastomeres, as well as in various porcine tissues and canine myocardium (Bustin, 2000).

4.6.4 Data analysis

All the reactions were performed in duplicates as suggested by Nolan *et al.* (2006). Nolan *et al.* (2006) indicated that reactions carried out in duplicate are generally sufficient as triplicate is needed to perform only if Cts are > 35. All replicates should be within 0.5 Ct of each other. The reactions should be repeated if the data of the replicates differ by > 0.5 Ct. The minimum difference of 5 Ct between the NRTC and sample data point must be ensured. The reactions should be repeated if the data between the NRTC and sample differ < 5 Ct.

All the reactions were run for 35 cycles. This is because above 35 cycles the variability will be greater and quantification may be unreliable (Nolan *et al.*, 2006). It is also important to ensure that the dynamic range spans the Ct values anticipated for experimental analysis (example: Figure 3.3). It is not valid to extrapolate quantification values of test samples beyond the values of the samples in the dilution series tested.

Relative quantitation using two standard curves method

The term relative quantitation is used when two or more genes are compared to each other with the result being a ratio. An endogenous control or a housekeeping gene is normally compared to a gene of interest. There are several ways of doing relative quantitation, such as relative quantitation using two standard curves and comparative ΔΔCt method. The main difference between absolute and relative quantitation is that absolute quantitation results in an output amount of copy numbers while relative quantitation uses relative numbers (e.g. fold differences) where the results are given as a ratio. The result is the comparison between the ratios.

In the present study, relative standard curve method was used to quantitate alterations in mRNA levels using RT-qPCR. The standard curve method is the most common approach to determine relative quantification. Analysis of the data from the standard curve can provide a substantial amount of information of the assay. Relative quantification determines the changes in steady-state transcription of a gene and is often adequate.

4.6.5 Expression level of apoptosis-related genes in the cytotoxic active extract-treated cells

In order to determine the expression level of apoptosis-related genes induced in the cytotoxic active extract-treated cells, the mRNA levels of p53, caspase-3 and c-myc were evaluated by two-step RT-qPCR. The mRNA level of ß-actin was used as internal control for template levels. Only extracts which were active in cytotoxic activity and detected with DNA fragmentation were tested in the gene expression study. Concentration of the extract needed to achieve 50 % growth inhibition (IC_{50}) was used to stimulate the cells over the period of 6 h. The qPCR assay which has been optimized (section 4.6.2) was applied to the experiment set in the present study.

(i) Expression level of apoptosis-related genes in the *P. bleo* cytotoxic active extract-treated cells

Methanol extract

Figure 4.32 show the time dependency effects of mRNA levels of p53, caspase-3 and c-myc in KB cells induced with methanol extract of *P. bleo*. The expression of p53, caspase-3 and c-myc were highest at 30 min, 15 min and 6 h, respectively. The mRNA expression level of caspase-3 was markedly increased when the KB cells were treated with the methanol extract of *P. bleo* at 15 min (4.9-fold increase as compared to control cells) and significantly ($p < 0.05$) decreased 1.5- to 1-folds after treatment for 30 min to 3 h. Interestingly, the caspase-3 expression level was increased again at 6 h (1.9-fold increase as compared to control cells). Generally, a significant increase ($p < 0.05$) level of c-myc expression was observed in KB cells cultured in the presence of methanol extract of *P. bleo* compared to control, except a drastic drop at 1 h. Meanwhile, the expression level of p53 mRNA was slightly increased at 30 min (1.5-

fold increase as compared to control cells), and significantly decreasing ($p < 0.05$) thereafter, to the level below the baseline.

Thus, the results strongly indicate that the methanol extract of *P. bleo* killed the KB cells through apoptosis mechanism mainly *via* the activation of caspase-3 and c-myc while the role of p53 cannot be ruled out. The activation of caspase-3 has been reported to be important in mediating some of the apoptotic pathways (Janicke *et al*., 1998). The c-myc protein is also important in the induction of apoptosis (Evans *et al*., 1992). These findings were in agreement with the study of Tan *et al.* (2005) and Er *et al.* (2007) on methanol extract of *P. bleo*. In the study done by Tan *et al.* (2005), the authors showed that the levels of caspase-3 and c-myc proteins were elevated in the T-47D cells (human breast cancer cell line) treated with the methanolic extract of the leaves of *P. bleo*. However, the study was carried out using conventional semi-quantitative RT-PCR which is not accurate enough while the current study was carried out using RT-qPCR assay. Nevertheless, Er *et al.* (2007) reported that there was an increase in the level of apoptosis in 4T1 (mouse mammary cancer cell line) and NIH/3T3 (normal mouse fibroblast cell line) cells treated with increasing concentrations of the *P. bleo* extract. Thus, the current study confirmed the result of Tan *et al.* (2005) and Er *et al.* (2007).

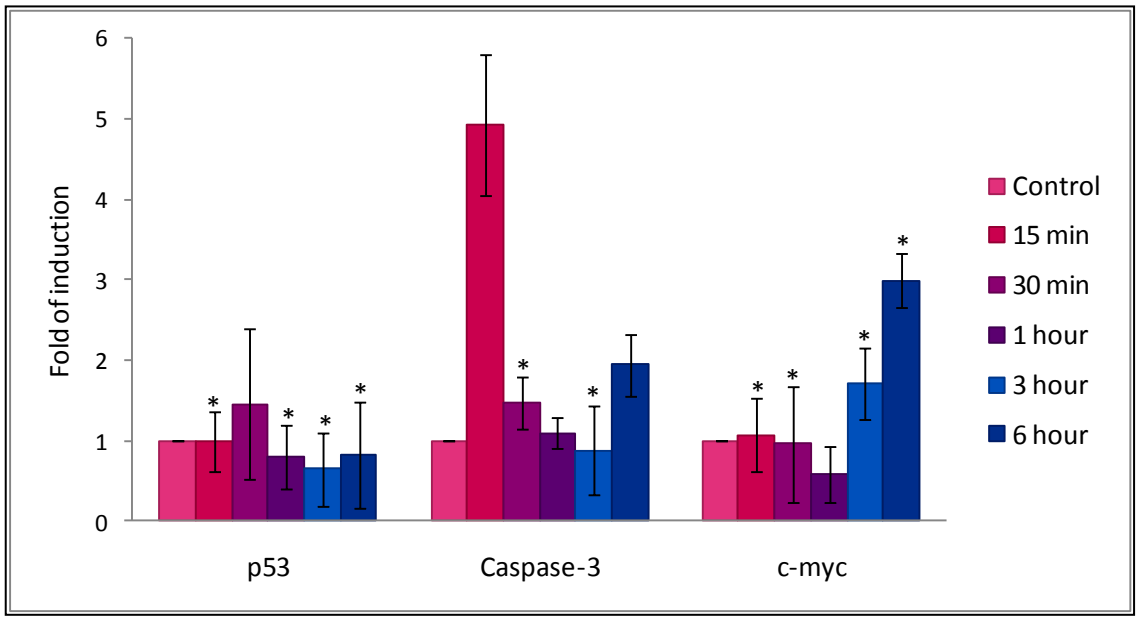


Figure 4.32: The mRNA expression of p53, caspase-3 and c-myc detected in KB cells treated with methanol extract of *P. bleo* for different durations. Results are expressed as means ± standard deviation. *: Significant difference from the control ($p < 0.05$).

Ethyl acetate extract

Figure 4.33 shows the time dependency effects of p53, caspase-3 and c-myc mRNA levels in KB cells induced with ethyl acetate extract of *P. bleo*. The expression of p53, caspase-3 and c-myc were highest at 1 h, 3 h and 6 h, respectively. A significant increase ($p < 0.05$) of p53 expression was observed in the KB cells treated with ethyl acetate extract of *P. bleo* at 15 min (4.7-fold increase as compared to control cells). However, the expression level of p53 was significantly reduced ($p < 0.05$) to 3.6-fold after 30 min incubation and significantly increased ($p < 0.05$) again to 4.8-fold at 1 h, and decreasing thereafter.

Whilst, the expression level of caspase-3 was slightly increased at 15 min (1.7-fold increase as compared to control cells), then decreased at 30 min and maintained approximately the same level thereafter (2.8-, 2.9- and 2.7-folds as compared to control

cells at 1 h, 3 h and 6 h, respectively). There was an gradually increase in the c-myc expression observed in the KB cells with the increasing of incubation time, with the highest level of c-myc expression observed at 6 h (2-fold increase as compared to control cells).

Thus, the results here indicate that the apoptosis elicited by the ethyl acetate extract on KB cells was mediated largely *via* p53 although the role of caspase-3 and c-myc cannot be ruled out. Previous studies have showed that tumour suppressor protein p53 was an important molecule for apoptosis and cell cycle arrest (May and May, 1999). Studies to date have also demonstrated that many natural phytochemicals could induce apoptosis and cell cycle arrest in p53 pathway (Ayyoubi and Muhtasib, 2007; Roemer *et al*., 2001; Pillai *et a*l., 2004). The significantly high expression of p53 in KB cells induced with ethyl acetate extract of *P. bleo* could be due to the presence of bioactive compound(s) in the leaves that can induce apoptosis in the cells.

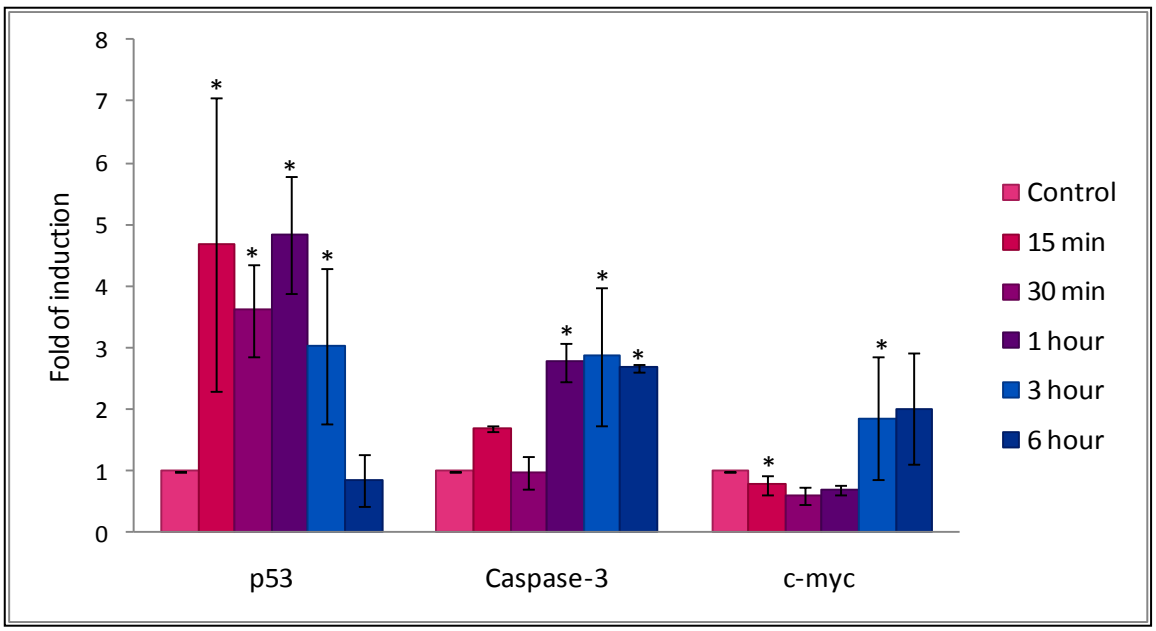


Figure 4.33: The mRNA expression of p53, caspase-3 and c-myc detected in KB cells treated with ethyl acetate extract of *P. bleo* for different durations. Results are expressed as means ± standard deviation. *: Significant difference from the control ($p < 0.05$).

(ii) Expression level of apoptosis-related genes in the *P. grandifolia* cytotoxic active extract-treated cells

Hexane extract

Figure 4.34 show the time dependency effects of p53, caspase-3 and c-myc mRNA levels in KB cells induced with hexane extract of *P. grandifolia*. The expression of p53, caspase-3 and c-myc were highest at 6 h, respectively. It is noteworthy to mention that the trend of p53, caspase-3 and c-myc mRNA expression levels in KB cells induced with hexane extract of *P. grandifolia* was similar. The mRNA expression levels of p53, caspase-3 and c-myc were lower than the control at incubation time of 15 min to 3 h and increased drastically when the cells were treated with the extract at 6 h (2.6-, 2.5- and 3.9-folds increase as compared to the control cells, respectively). However, the statistical analysis did not show any significant difference in the expression level of p53 compared to the control. Thus, the results suggest that the apoptotic effects of the hexane extract of *P. grandifolia* in the KB cells were associated with the increase in p53, caspase-3 and c-myc activation.

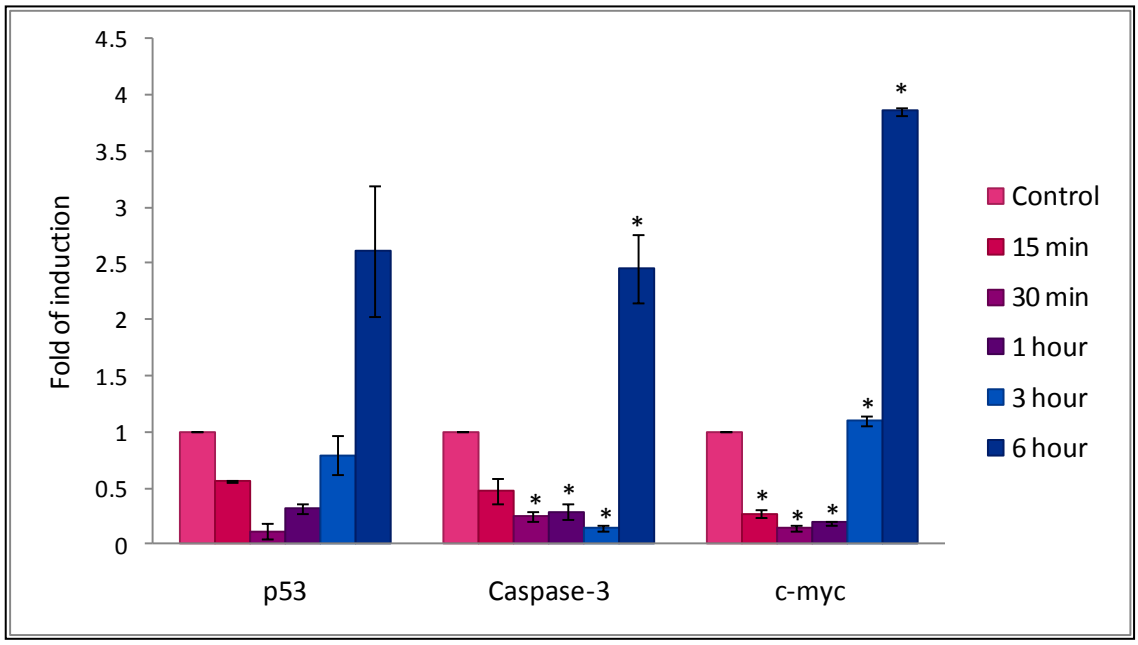


Figure 4.34: The mRNA expression of p53, caspase-3 and c-myc detected in KB cells treated with hexane extract of *P. grandifolia* for different durations. Results are expressed as means ± standard deviation. *: Significant difference from the control ($p < 0.05$).

Ethyl acetate extract

a) KB cell line

Figure 4.35 shows the time dependency effects of p53, caspase-3 and c-myc mRNA levels in KB cells induced with ethyl acetate extract of *P. grandifolia*. The expression of p53, caspase-3 and c-myc were highest at 6 h, 15 min and 6 h, respectively. The trend of p53 and c-myc mRNA expression levels was similar. The mRNA expression levels of p53 and c-myc were lower than the control at incubation time of 15 min to 3 h and increased drastically when the cells were treated with the extract at 6 h (13.1- and 4.7-folds increase as compared to the control cells, respectively). Meanwhile, the expression level of caspase-3 was significantly increased ($p < 0.05$) after 15 min incubation with the extract but decreasing thereafter to the level below control and increased again at 6 h (4.7-fold increase as compared to the control).

In summary, the significant increase ($p < 0.05$) of p53 expression (13.1-fold increase as compared to the control at 6 h) in contrast to the mild increase of caspase-3 and c-myc expression after exposure to the extract indicated that the ethyl acetate extract of *P. grandifolia* killed the KB cells through apoptosis mechanism mainly *via* the activation of p53 whereas the role of p53 and caspase-3 cannot be ruled out.

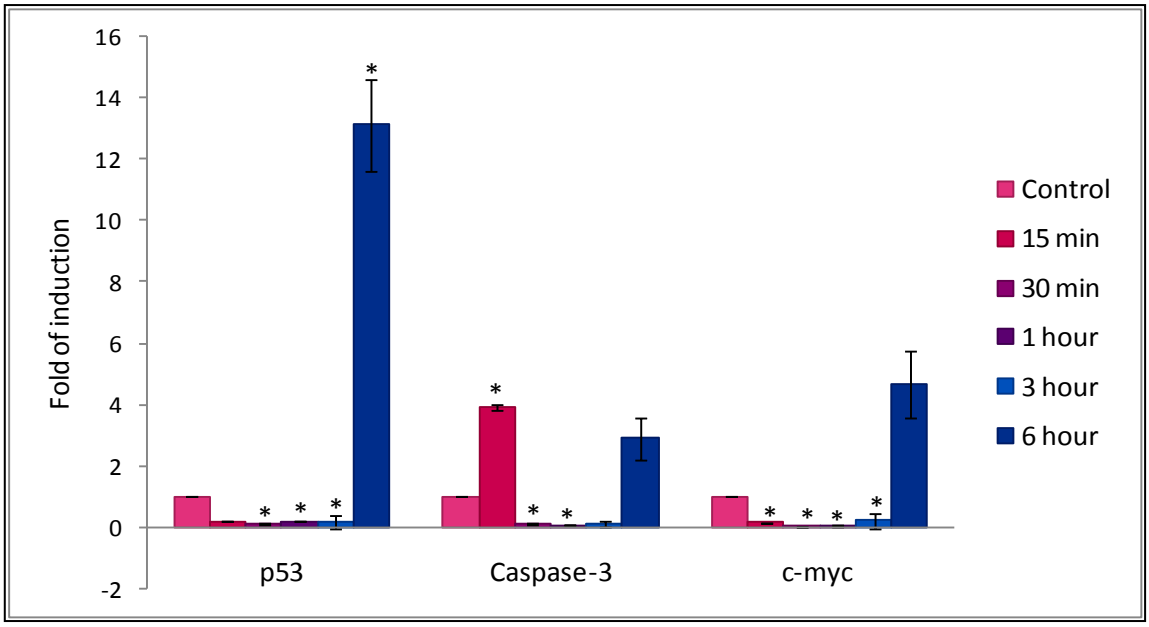


Figure 4.35: The mRNA expression of p53, caspase-3 and c-myc detected in KB cells treated with ethyl acetate extract of *P. grandifolia* for different durations. Results are expressed as means ± standard deviation. *: Significant difference from the control ($p < 0.05$).

b) MCF7 cell line

The time dependency effects of p53, caspase-3 and c-myc mRNA levels in MCF7 cells induced with ethyl acetate extract of *P. grandifolia* is shown in Figure 4.39. The expression of p53, caspase-3 and c-myc were highest at 15 min, respectively. Figure 4.36 shows that the steady state mRNA levels of p53 and caspase-3 were increased when the MCF7 cells were treated with the extract at 15 min (1.5- and 3.2-

folds increase as compared to control cells, respectively). However, the expression levels of p53 and caspase-3 were decreasing thereafter, to the level below control.

Whilst, the expression level of c-myc was significantly increased ($p < 0.05$) when the cells were treated with the extract at 15 min (4.3-fold increase as compared to control cells), but dropped to 1.7-fold after 30 min incubation and increased again to 2-fold at 1 h. In summary, the results here indicate that the ethyl acetate extract of *P. grandifolia* killed the MCF7 cells through apoptosis mechanism *via* the activation of c-myc.

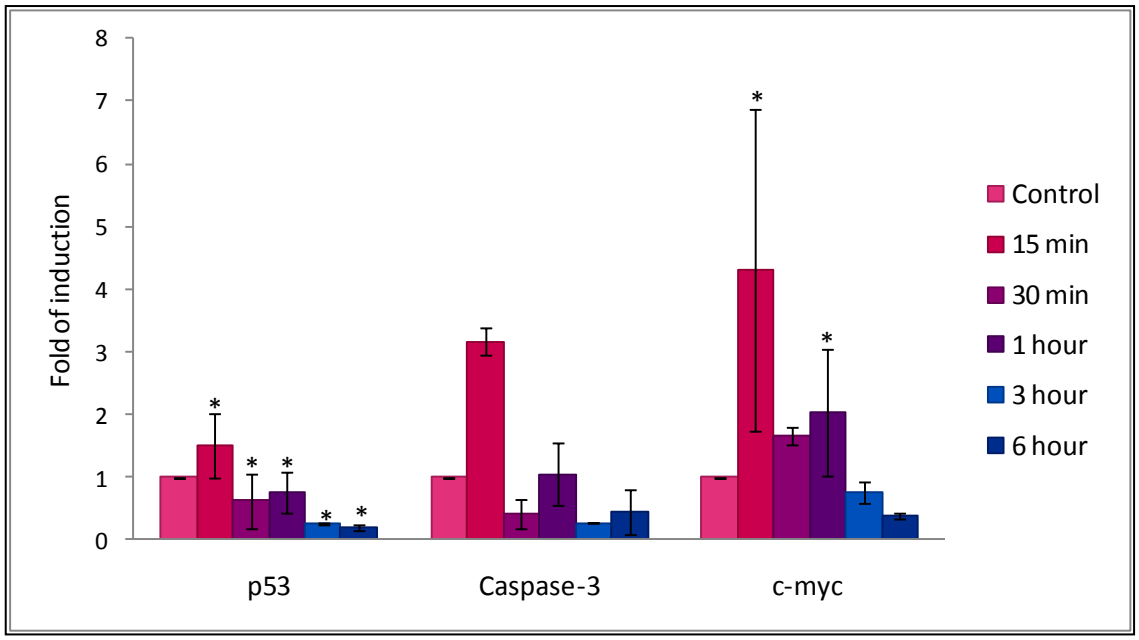


Figure 4.36: The mRNA expression of p53, caspase-3 and c-myc detected in MCF 7 cells treated with ethyl acetate extract of *P. grandifolia* for different durations. Results are expressed as means ± standard deviation. *: Significant difference from the control ($p < 0.05$).

(iii) Summary of expression level of apoptosis-related genes in the *Pereskia spp.* cytotoxic active extract-treated cells

In summary, LUX RT-qPCR assay offers significant advantages with respect to the rapidity, sensitivity and reproducibility of quantification assay for gene expression.

The protocol for the evaluation of mRNA expression levels of apoptosis-related genes using LUX RT-qPCR assay developed in this study can be recommended for gene expression study involving natural products.

The LUX RT-qPCR analysis [section 4.6.5 (i) and 4.6.5 (ii)] showed the mRNA expression levels of c-myc were markedly increased in the cancer cells treated with the cytotoxic active extracts while the p53 and caspase-3 expression were only slightly increased as compared to c-myc in all cases, except for the *P. grandifolia* ethyl acetate extract in KB cells. The apoptotic effects of the *P. grandifolia* ethyl acetate extract in KB cells were associated with increases in all three c-myc, p53 and caspase-3 activation.

The results obtained here suggest that the cytotoxic active extracts of *P. bleo* and *P. grandifolia* contain bioactive compounds capable of inducing apoptosis in selected cancer cell lines.

4.7 Acute oral toxicity assessment of *P. bleo* and *P. grandifolia* crude extracts

Investigation of the acute toxicity is the first step in the toxicological analysis of herbal drugs (Deciga-Campos *et al.*, 2007). Acute oral toxicity was undertaken in the present study to determine the safety parameters of the leaves of *P. bleo* and *P. grandifolia*. Mortality, clinical signs, gross findings and body weights of mice were observed and measured for 14 days after the oral administration of crude methanol extracts of both *Pereskia spp.* The crude methanol extracts were used in this acute oral toxicity study as consumers normally consume the whole plant leaves.

Table 4.19 showed the results of the acute toxicity of the crude extracts of *P. bleo* and *P. grandifolia*. There were no deaths for all doses of crude methanol extracts

of *P. bleo* and *P. grandifolia* tested. Throughout the 14 days observation period, all the mice did not show changes in behavior (i.e. ataxia, hyperactivity, hypoactivity) or produce any variations in the general appearance, and gained weight with no adverse clinical signs of toxicity at any doses.

Since there were no deaths for all doses tested for crude methanol extracts of *Pereskia bleo* and *Pereskia grandifolia* (the LD_{50} values of crude *P. bleo* and *P. grandifolia* extracts were > 2500 mg/kg); this indicated that both *Pereskia spp.* did not cause acute toxicity. According to the chemical labeling and classification of acute systemic toxicity based on oral LD_{50} values which was recommended by globally harmonized system of classification (GHS) (OECD, 1998), crude extracts of both *Pereskia spp.* are assigned to class 5 (LD_{50} > 2000 mg/kg), which are termed as lowest toxicity class (no label; *unclassified*).

In summary, the result was in agreement to that of *in vitro* experiments, whereby crude methanol extracts of *P. bleo* and *P. grandifolia* did not show cytotoxicity against normal MRC-5 cells (Table 4.17). Based on the outcome of acute toxicity, the crude extracts of both *Pereskia spp.* can be regarded safe in experimental mice. However, the crude extracts of *P. bleo* may not be safe to consuming over a long period of time as there was a report that *P. bleo* has mutagenice effects (Er *et al*., 2007). Thus, further studies on the mutagenic and toxicity effect over a longer period of time involving detection of effects on vital organ functions should be carried out to ensure that the plants are safe for human consumption.

Table 4.19: Results of the potential toxic effect of the crude extracts of *P. bleo* and *P. grandifolia* in mice

Plants	Dose (mg/kg)											
	0[a]		500		1000		1500		2000		2500	
	M	F	M	F	M	F	M	F	M	F	M	F
P. bleo	0/5[b]	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
P. grandifolia	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

M: male ICR mice; F: female ICR mice

[a]control group (treatment without extract) [b]Number of animals dead/number of mice used.

4.8 Chemical constituents from the bioactive extracts of *P. bleo* and *P. grandifolia*

The separation and purification of chemical constituents were carried out using one or a combination of several chromatographic techniques, such as column chromatography, TLC (Thin Layer Chromatography) and prep-TLC (preparative-TLC). The choice of techniques depends mostly on the solubility and polarities of the compounds to be separated.

Column chromatography was used first to separate large scale extract. The choice of the size and length of the column depends on the amount of the extract used. Approximately equal volume was collected for each fraction. These fractions were then concentrated using rotary evaporator and monitored for purity using TLC. The selection of a proper solvent is vitally important to separate the compounds from its crude. Fractions with similar composition were combined. If a single spot was seen on the TLC, purification was carried out, which sometimes needs to be done two or three times (or more) in order to obtain pure compounds before it could be sent for further analysis. In some cases where the compounds were not pure, a proper solvent system was determined.

Technique such as prep-TLC was used sometimes for further separation by using TLC silica gel 60 F_{254} glass plates (20 cm x 20 cm; Merck). Separated compounds were recovered by scrapping off the appropriate zones on the developed plate, eluting with powder with suitable solvent. The solution was then concentrated using rotary evaporator and the purity of residue was checked using TLC. If a single substance is present, purification can be carried out to obtain pure compounds before it could be sent for further analysis.

4.8.1 Identification of compounds in hexane extract of *P. bleo* using GCMS

Four compounds were identified from the hexane extract of *P. bleo* by GCMS analysis (Appendix B), which were methyl palmitate (13.77 %), methyl linoleate (4.55 %), methyl α-linolenate (19.83 %) and phytol (57.99 %). The structures of the above compounds are illustrated in Figure 4.37.

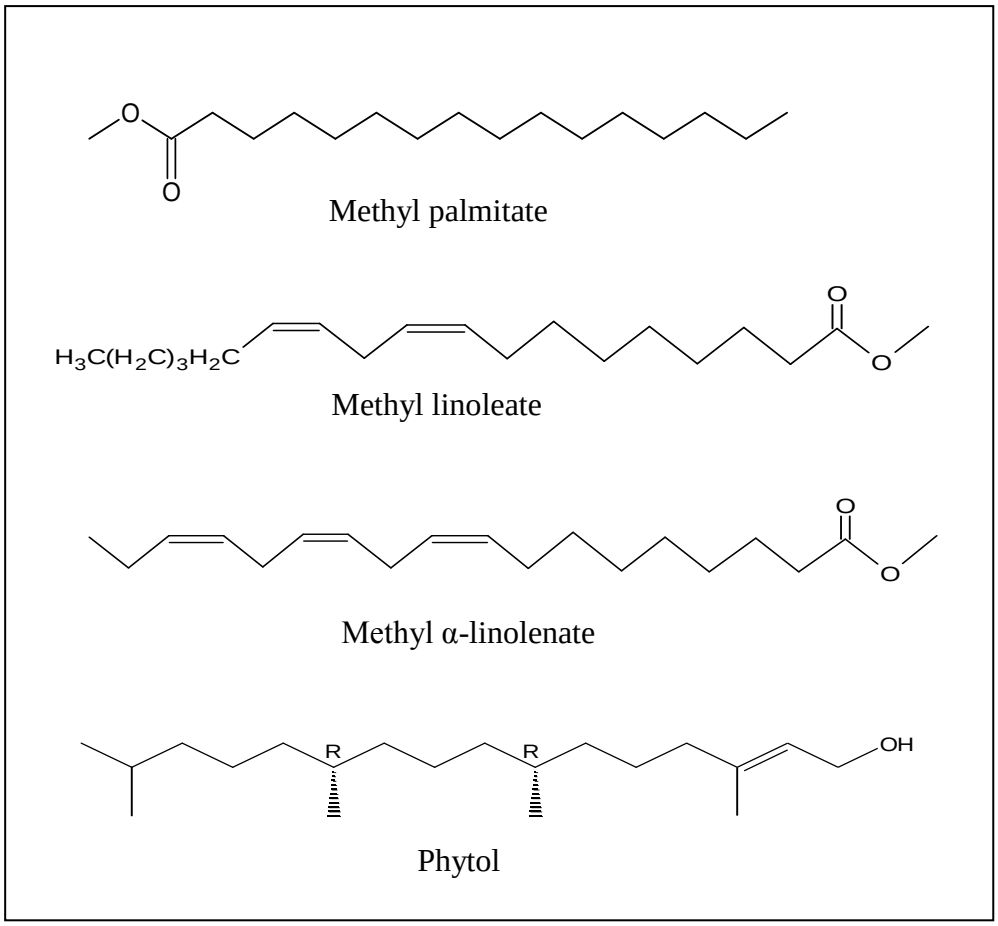


Figure 4.37: Compounds identified from the hexane extract of *P. bleo* using GCMS analysis

The mass-spectral data of the identified compounds in hexane extract of *P. bleo* are as follows.

EI-MS *m/z* (%) (methyl palmitate):

270 (3, $[M]^+$), 239 (2, $[M\text{-}OCH_3]^+$, $C_{16}H_{31}O_1^+$), 227 (8), 213 (1), 199 (2), 185 (4), 171 (4), 157 (2), 143 (16), 129 (8), 115 (2), 107 (1), 97 (8), 87 (70, $CH_2CH_2CO_2CH_3^+$), 74 (100, $C_3H_6O_2^+$), 65 (1), 55 (32).

The mass spectrum gave the molecular ion peak at *m/z* 270, which suggested a molecular formula to be $C_{17}H_{34}O_2$ (Appendix B1). The comparison with NIST mass-spectral library (NIST 05 MS Library, 2002) and Adams (2001) confirmed this compound as methyl palmitate (or methyl hexadecanoate).

EI-MS *m/z* (%) (methyl linoleate):

294 (3, $[M]^+$), 263 (2, $[M\text{-}OCH_3]^+$, $C_{18}H_{31}O_1^+$), 220 (1), 178 (2), 164 (6), 150 (10), 135 (11), 123 (12), 109 (25), 95 (58), 81 (82), 67 (100), 55 (86).

The mass spectrum gave the molecular ion peak at *m/z* 294, which suggested a molecular formula to be $C_{19}H_{34}O_2$ (Appendix B2). The comparison with NIST mass-spectral library (NIST 05 MS Library, 2002) and Adams (2001) confirmed this compound as methyl linoleate [or methyl (Z,Z)-9,12-octadecadienoate].

EI-MS *m/z* (%) (methyl α-linolenate):

292 (2, $[M]^+$), 264 (2), 249 (1), 236 (2), 222 (1), 207 (1), 199 (1), 191 (1), 181 (1), 173 (2), 163 (2), 149 (10), 135 (12), 121 (16), 108 (32), 95 (56), 87 (17), 79 (100), 67 (70), 55 (74).

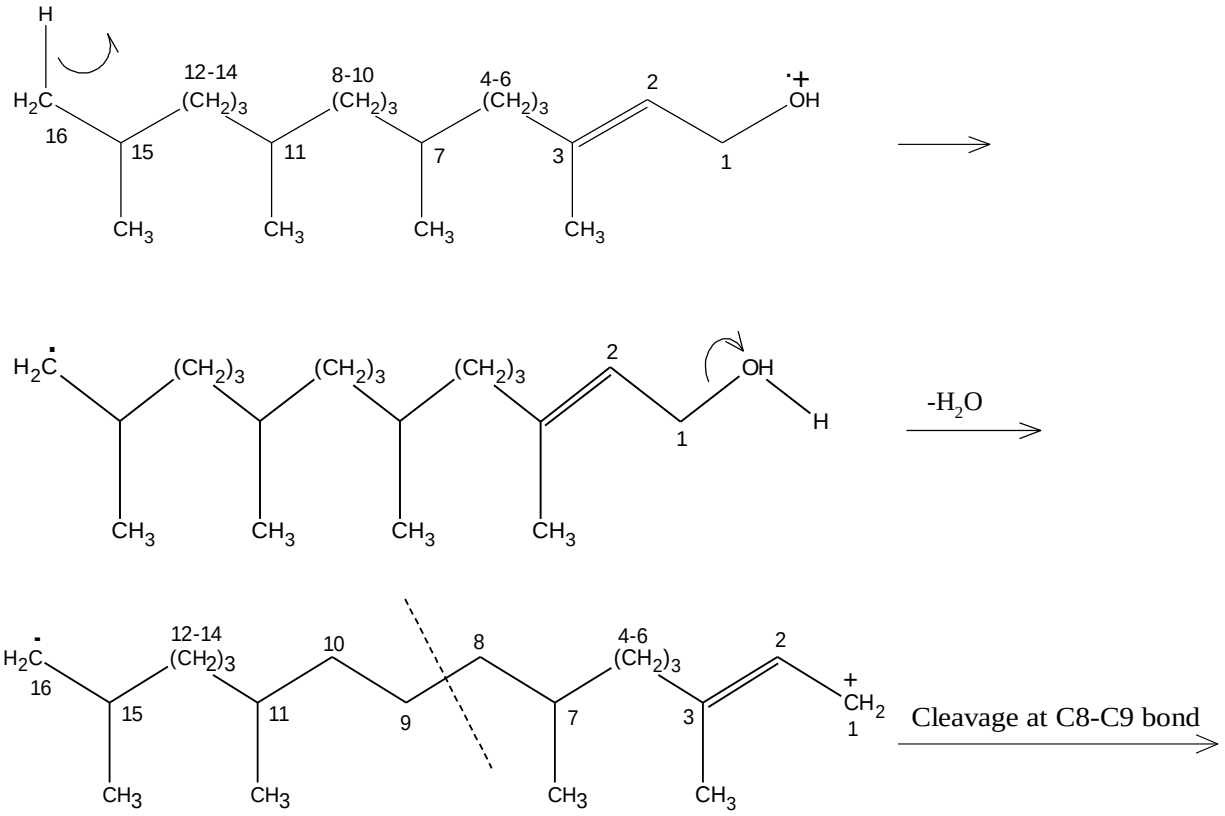
The mass spectrum gave the molecular ion peak at *m/z* 292, which suggested a molecular formula to be $C_{19}H_{32}O_2$ (Appendix B3). The comparison with NIST mass-spectral library (NIST 05 MS Library, 2002) and Adams (2001) confirmed this compound as methyl α-linolenate [or methyl (Z, Z, Z)-9,12,15-octadecatrienoate].

EI-MS *m/z* (%) (phytol):

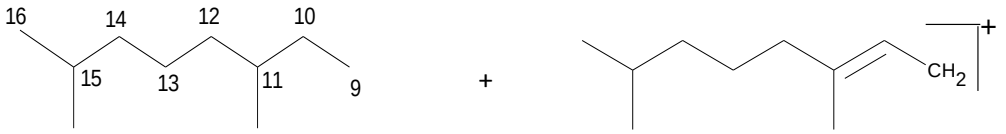
278 (2, $[M^+\text{-}H_2O]$), 137 (5, $[C_{10}H_{18}]^+$), 123 (40, $[M\text{-}CH_2]^+$), 109 (15, $[M\text{-}CH_2]^+$), 95 (36, $[M\text{-}CH_2]^+$), 81 (38, $[M\text{-}CH_2]^+$), 71 (100, $(CH_3)_2CHCH_2CH_2^+$), 57 (62, $(CH_3)_2CHCH_2^+$).

The mass spectrum of phytol (Appendix B4) did not show a molecular ion peak. However, a peak at *m/z* 278 was observed corresponding to loss of H_2O. This peak is most noticeable in the spectra of primary alcohols (Silverstein *et al.*, 1998). The peak at *m/z* 137 is consistent with the fragment ion $[C_{10}H_{18}]^+$.

The fragmentation of phytol can be rationalized as follows:



m/z = 278, $[C_{20}H_{38}]^+$



m/z = 137, $[C_{10}H_{18}]^+$

The mass spectrum of phytol gives a strong base peak at *m/z* 71 corresponding to the fragment ion $(CH_3)_2CHCH_2CH_2^+$.

4.8.2 Identification of compounds in hexane extract of *P. grandifolia* using GCMS

Four compounds were identified from the hexane extract of *P. grandifolia* by GCMS analysis (Appendix C), which were methyl palmitate (28.27 %), methyl linoleate (12.60 %), methyl α-linolenate (54.83 %) and methyl stearate (1.17 %). The structures of the above compounds are illustrated in Figure 4.38.

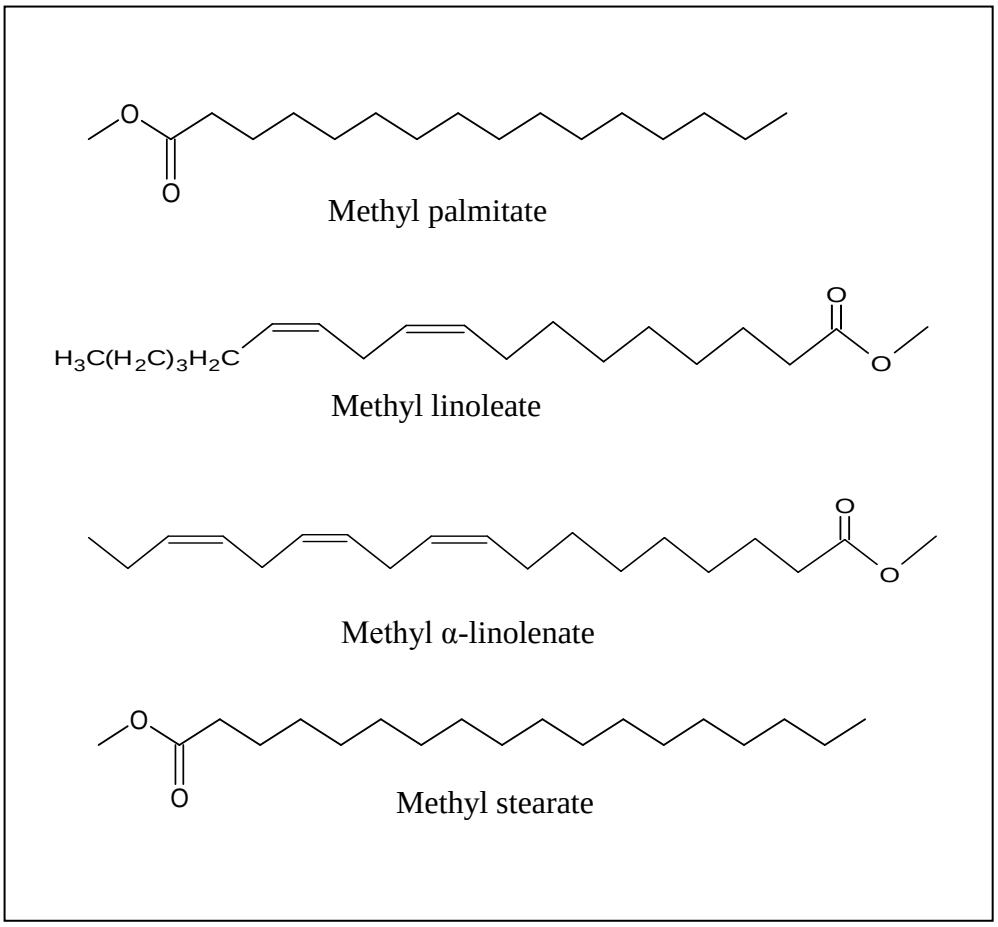


Figure 4.38: Compounds identified from the hexane extract of *P. grandifolia* using GCMS analysis

The mass-spectral data of the identified compounds in hexane extract of *P. grandifolia* are as follows.

EI-MS *m/z* (%) (methyl palmitate):

270 (3, $[M]^+$), 239 (2, $[M\text{-}OCH_3]^+$, $C_{16}H_{31}O_1^+$), 227 (8), 213 (1), 199 (2), 185 (4), 171 (4), 157 (2), 143 (16), 129 (8), 115 (2), 107 (1), 97 (8), 87 (70, $CH_2CH_2CO_2CH_3^+$), 74 (100, $C_3H_6O_2^+$), 65 (1), 55 (32).

The mass spectrum gave the molecular ion peak at *m/z* 270, which suggested a molecular formula to be $C_{17}H_{34}O_2$ (Appendix C1). A comparison with NIST mass-spectral library (NIST 05 MS Library, 2002) and Adams (2001) confirmed this compound as methyl palmitate (or methyl hexadecanoate).

EI-MS *m/z* (%) (methyl linoleate):

294 (3, $[M]^+$), 263 (2, $[M\text{-}OCH_3]^+$, $C_{18}H_{31}O_1^+$), 220 (1), 178 (2), 164 (6), 150 (10), 135 (11), 123 (12), 109 (25), 95 (58), 81 (82), 67 (100), 55 (86).

The mass spectrum gave the molecular ion peak at *m/z* 294, which suggested a molecular formula to be $C_{19}H_{34}O_2$ (Appendix C2). The comparison with NIST mass-spectral library (NIST 05 MS Library, 2002) and Adams (2001) confirmed this compound as methyl linoleate [or methyl (Z,Z)-9,12-octadecadienoate].

EI-MS *m/z* (%) (methyl α-linolenate):

292 (2, $[M]^+$), 264 (2), 249 (1), 236 (2), 222 (1), 207 (1), 199 (1), 191 (1), 181 (1), 173 (2), 163 (2), 149 (10), 135 (12), 121 (16), 108 (32), 95 (56), 87 (17), 79 (100), 67 (70), 55 (74).

The mass spectrum gave the molecular ion peak at *m/z* 292, which suggested a molecular formula to be $C_{19}H_{32}O_2$ (Appendix C3). The comparison with NIST mass-

spectral library (NIST 05 MS Library, 2002) and Adams (2001) confirmed this compound as methyl α-linolenate [or methyl (Z, Z, Z)-9,12,15-octadecatrienoate].

EI-MS *m/z* (%) (Methyl stearate):

298 (6, $[M]^+$), 267 (2), 255 (8), 241 (1), 227 (1), 213 (2), 199 (6), 185 (4), 143 (21), 129 (6), 111 (3), 97 (8), 87 (69, $CH_2CH_2CO_2CH_3^+$), 74 (100, $C_3H_6O_2^+$), 55 (30).

The mass spectrum gave the molecular ion peak at *m/z* 298, which suggested the molecular formula to be $C_{19}H_{38}O_2$ (Appendix C4). The comparison with NIST mass-spectral library (NIST 05 MS Library, 2002) and Adams (2001) confirmed this compound as methyl stearate (methyl octadeacanoate).

Structural Determination of methyl ester

The molecular ion peak of a methyl ester is usually distinct and prominent. All the methyl esters identified in the present study displayed the molecular ion peak. Mass spectrums of all the methyl esters show peak M^+-OCH_3 which represented the fragment ion $R—C\equiv O^+$. The ion $R—C\equiv O^+$ gives an easily recognizable peak for esters and occurs at M^+- OCH_3 in methyl esters (Silverstein *et al.*, 1998).

For methyl palmitate and methyl stearate, the molecular ion peak is mostly weak in the range *m/z* 130 to ~200, but becomes somewhat more intense beyond this range. For saturated fatty ester, the most characteristic peak results from McLafferty rearrangement and cleavage one bond removed from the C=O group. Thus, methyl palmitate and methyl stearate unbranched at the α carbon gives a strong base peak at *m/z* 74 which formally represented by the ion $C_3H_6O_2^+$ results from McLafferty rearrangement (Silverstein *et al.*, 1998). Mass spectrum of methyl palmitate and methyl stearate showed peak (*m/z* 87) which formally represented by the ion

$[CH_2CH_2CO_2CH_3]^+$ is always more intense than its homologs. McLafferty rearrangement of methyl palmitate is showed in Figure 4.39.

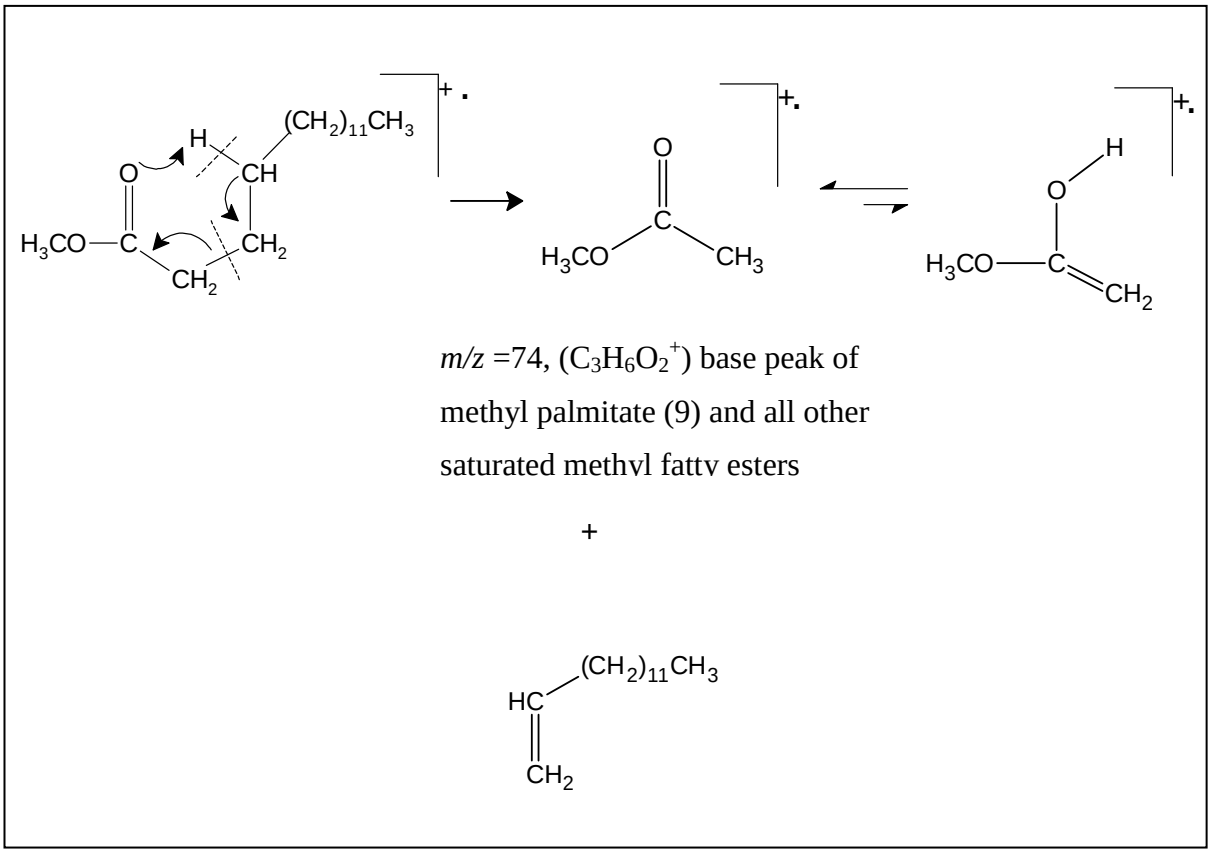


Figure 4.39: McLafferty rearrangement of methyl palmitate

4.8.3 Chemical constituents from the bioactive ethyl acetate extract of *P. bleo*

On repeated chromatographic purification of the active ethyl acetate fraction, five compounds and a mixture were isolated and identified as 2,4-di-tert-butylphenol (**1**), α-tocopherol (**2**), phytol (**3**), ß-sitosterol (**4**), dihydroactinidiolide (**5**) and a **mixture A** which consisted of campesterol (**6**), stigmasterol (**7**) and β-sitosterol (**4**). All the compounds have not been reported for this plant. The structures of compounds **1-7** are illustrated in Figure 4.40.

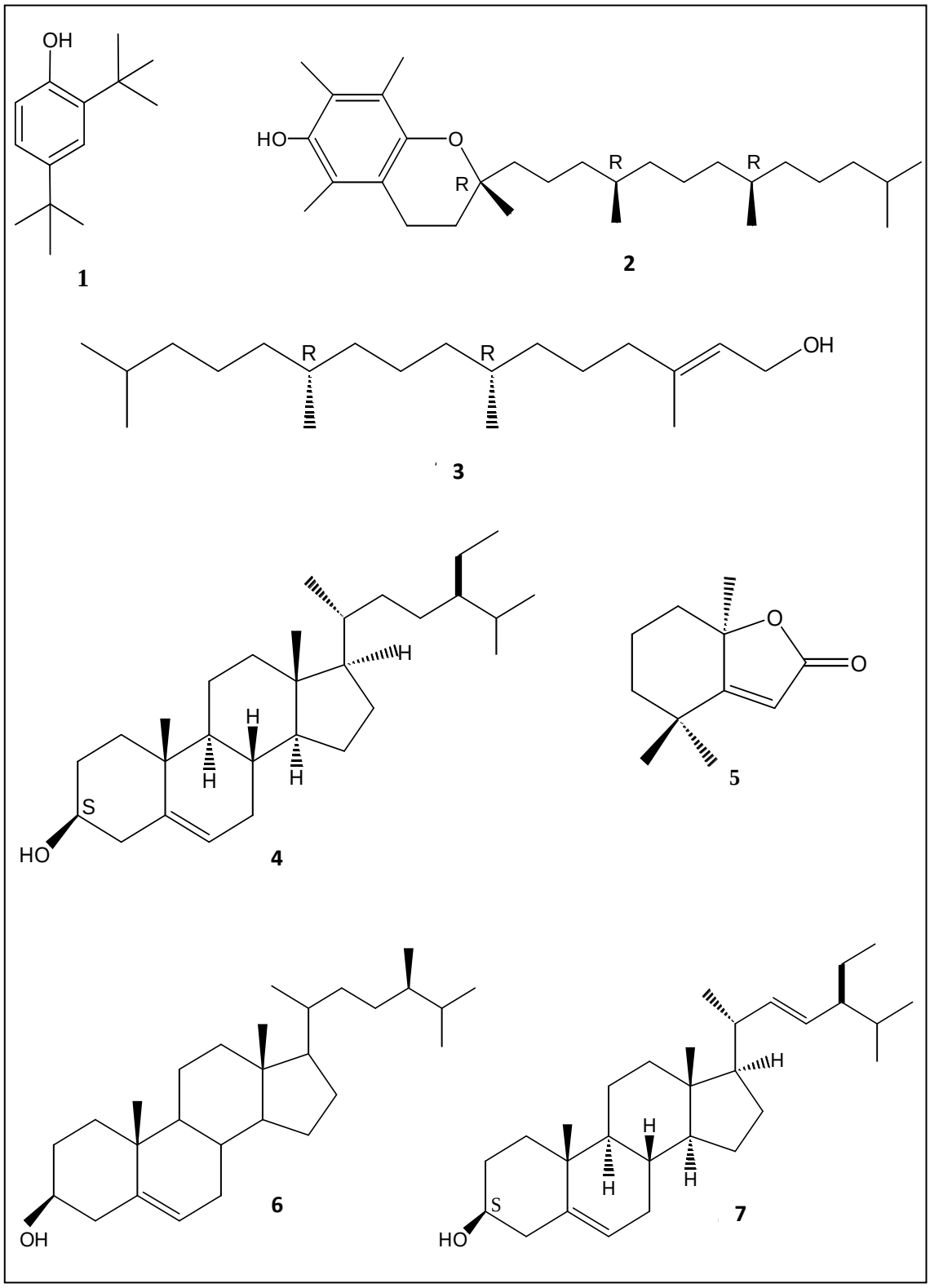
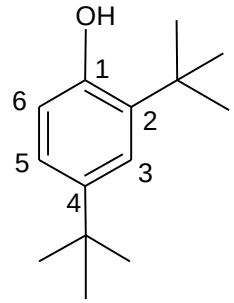


Figure 4.40: Chemical constituents from the bioactive ethyl acetate extract of *P. bleo*

(i) Structural determination of 2,4-ditert-butylphenol (1)



1

The mass spectroscopic and the NMR spectral data obtained for compound **1** are shown as follows:

EI-MS *m/z* (%):

206 (15, $[M]^+$), 191 (100, $[M- CH_3]^+$), 175 (4), 163 (6), 57 (32).

^{1}H-NMR ($CDCl_3$, 399.65 MHz) δ:

1.24 (9H, s, three methyls), 1.36 (9H, s, three methyls), 6.53 (d, J=8.39 Hz, H-6), 7.02 (dd, *J*=8.39, 2.40 Hz, H-5), 7.24 (d, *J*=2.40 Hz, H-3).

^{13}C-NMR ($CDCl_3$, 100.40 MHz) δ:

151.7 (C-1), 135.2 (C-2), 123.5 (C-3), 143.0 (C-4), 124.0 (C-5), 115.9 (C-6), 30.0 (C-7), 34.7 (C-8), 31.6 (C-9, 10, 11), 31.8 (C-12, 13, 14).

Compound **1** obtained as yellow-coloured crystalline powder was identified as 2,4-di-tert-butylphenol from GC-MS analysis (NIST 05 mass-spectral library, 2002) and comparison with published data (Kamitori *et al.*, 1984). The mass spectrum gave a molecular ion peak at *m/z* 206, which was consistent with the molecular formula of

$C_{14}H_{22}O$ (Appendix D). The spectrum also showed that a methyl group is lost much more readily than an α hydrogen at *m/z* 191 consistent with the fragment ion $[M\text{-}CH_3]^+$.

The H-, ^{13}C- NMR and DEPT (Distortionless Enhancement by Polarisation Transfer) data (Appendix D1, D2 and D3) also support the existence of 2,4-di-tert-butylphenol. The H-NMR showed the presence of two methyl singlet peaks at δ1.24 and δ1.36 integrating for 9 protons each. The doublet at δ6.53 (J=8.39 Hz) was assigned to the aromatic H-6 proton. The H-5 proton was consistent with the peaks centred at δ7.02 which appeared as dd (*J*=8.39, 2.40 Hz). The peak at δ 7.24 (d, *J*=2.40 Hz) assigned to H-3 was coupled to H-5 with coupling constant of 2.40 Hz. The ^{13}C-NMR spectrum showed the presence of 14 carbons with two methyl C peaks integrating for six carbons, one C-O, three aromatic methyl, two quaternary and two quaternary aromatic carbon peaks. The NMR spectra thus support the identification of compound **1** as 2,4-di-tert-butylphenol.

2,4-Di-tert-butylphenol **(1)** is an antioxidant widely used in the plastic industries, and its presence in plants cannot readily be explained biogenetically. It is highly probable that the plant accumulated this compound from the soil it grew in, that might have contained the compound. In our experience, this compound has also been detected in other plants like *Termitomyces heimi*, *Pleurotus sajor-caju* and *Hericium erinaceus* collected from different locations to where the *P. bleo* leaves were obtained (unpublished data from our group of researchers working on *Termitomyces heimi*, *Pleurotus sajor-caju* and *Hericium erinaceus*). The presence of 2,4-di-tert-butylphenol (**1**) in our study is not an isolated case, as it has also been reported to exist in natural sources by other researchers (Yoon *et al*., 2006; Nogueira *et al*., 2001; Rana and Blazquez, 2007). To support our finding that 2,4-di-tert-butylphenol (**1**) is not an artifact, an extraction on *P. bleo* was repeated using redistilled methanol and ethyl

acetate. GC-MS analysis on the ethyl acetate extract still showed the presence of 2,4-di-tert-butylphenol (**1**) representing one of the component in the total ethyl acetate extract. This shows that 2,4-di-tert-butylphenol (**1**) is present in the extract itself and not a solvent artifact.

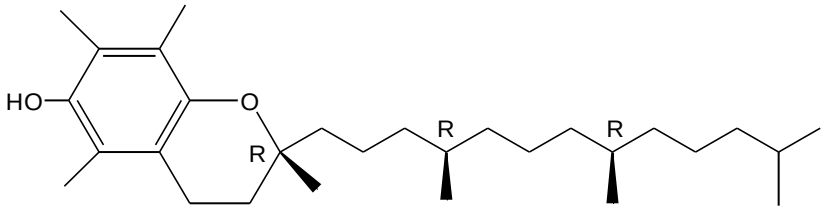
2,4-Di-tert-butylphenol (**1**) is structurally related to the well known antioxidant BHA (butylated hydroxyanisole). Phenolic antioxidants were reported to have cytoctoxic activity against cancer cells (Russo *et al*., 2009; Kozubek and Tyman, 1999) and exert anticarcinogenic activity, presumably through the induction of phase II detoxifying enzymes such as glutathione S-transferases and quinone reductase, which provides prevention of tumour initiation (Yoshioka *et al*., 1995; Yu *et al*., 1997). Phenolic antioxidants can also decrease oxidative stress-induced carcinogenesis by direct scavenging of ROS (Reactive Oxygen Species) (Tanaka *et al*., 1997) and by inhibiting cell proliferation secondary to the inhibition of protein phosphorylation (Schreck *et al*, 1992). In addition, phenolic compounds were reported to pose antimicrobial activity (Mitova *et al*., 2003).

Cytotoxic studies by Kadoma *et al.* (2009), Fujisawa *et al.* (2004) and Saito *et al.* (2001) also showed that 2,4-di-tert-butylphenol (**1**) was cytotoxic active against HSC-2 cells (human oral carcinoma cell line), HSG cells (human submandibular gland carcinoma cell line) and HGF cells (human gingival fibroblasts cell line). Furthermore, Yoon *et al.* (2006) reported that 2,4-di-tert-butylphenol (**1**) exhibited antioxidant activities on copper-mediated oxidation (IC_{50} value of 8.2 μM), AAPH-mediated oxidation (IC_{50} value of 9.9 μM) and SIN-1 mediated oxidation (52 %) in the TBARS assay. Kadoma *et al.* (2009) and Yoon *et al.* (2006) also reported that 2,4-di-tert-butylphenol (**1**) showed strong radical scavenging activity in DPPH assay with IC_{20} value of 1.01 mM.

In addition, a study on the uterotrophic effect of 2,4-di-tert-butylphenol (**1**) tested in ovariectomized CD1 mice had been conducted by Yoshimitsu *et al.* (2006). These authors made the point that there is no uterotrophic effect observed as no obvious changes in uteri and vagina were noticed in 2,4-di-tert-butylphenol (**1**) treated mice.

It can thus be assumed that 2,4-di-tert-butylphenol (**1**) may be one of the constituents that contribute to the reducing capacity (section 4.2.1), antioxidant activity (section 4.2.2-4.2.4), antimicrobial activity (section 4.3) and cytotoxic activity (section 4.4) of the ethyl acetate extract of *P. bleo*.

(ii) Structural determination of α-tocopherol (2)



2

The mass spectrum fragmentation obtained for compound **2** ia shown as follows:

EI-MS *m/z* (%):

430 (34, [M^+]), 205 (8, $C_{13}H_{17}O_2^+$), 165 (100, $C_{10}H_{13}O_2^+$).

Compound **2** was obtained as yellow viscous oil and identified as α-tocopherol through the GC-MS analysis (NIST 05 mass-spectral library, 2002) and comparison with published data (Pereira *et al*., 2002). The mass spectrum gave a molecular ion peak at *m/z* 430, consistent with the molecular formula of $C_{29}H_{50}O_2$ (Appendix E).

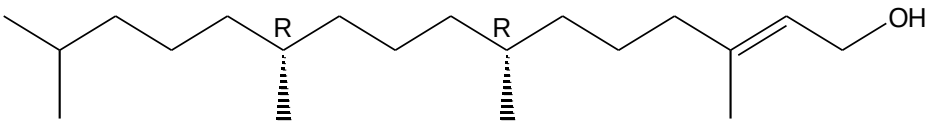
Vitamin E, a potent antioxidant, is one of the best quenchers for singlet oxygen, and can act as a chain-breaking antioxidant. It occurs in nature in ≥ 8 structurally related forms, i.e. four forms of tocopherols (α, ß, ɤ, δ) and four types of tocotrienols (α, ß, ɤ, δ). Among the various forms of vitamin E that have been identified, α-tocopherol (**2**) is the form that has been extensively studied and is present in most supplements (Jiang *et al*., 2001).

α-Tocopherol (**2**) is a dietary anti-oxidant capable of eliminating free radical damage, inducing apoptosis and decreasing oncogene expression (Bermudez *et al*., 2007). It is regarded as an intracellular antioxidant and is found to protect cells from carcinogenic chemicals due to its activity in inhibiting the peroxidation of polyunsaturated fatty acids and its damaging free-radical-mediated consequences (Choi and Lee, 2009; Jiang *et al*., 2001). Previous studies indicated that α-tocopherol decreases the toxicity of chemotherapy without reducing its effectiveness (Lamson and Brignall, 2003; Drisko *et al*, 2003).

Bermudez *et al*. (2007) suggested that α-tocopherol (**2**) might be an important protective agent against ovarian cancer cell growth as well as potentially effective therapeutic adjuvant. α-Tocopherol (**2**) has also been suggested to prevent prostate cancer (Bektic *et al*., 2005; Kirsh *et al*., 2006; Kampa *et al*., 2000; Zang *et al*., 2000), skin cancer (Reddy *et al*., 2003) and renal cell carcinoma (Wu *et al*., 2009). Several studies have confirmed α-tocopherol (**2**) as an anticancer agent with the animal studies (Steele *et al.*, 1994; Jiang *et al*., 2001).

Therefore, α-tocopherol (**2**) may be one of the constituents that contributed to the reducing capacity (section 4.2.1), antioxidant (section 4.2.2-4.2.4), and cytotoxic activities (section 4.4) of the ethyl acetate extracts of *P. bleo*.

(iii) Structural determination of phytol (3)



3

The EI-MS and the NMR spectral data obtained for compound **3** are shown as follows:

EI-MS *m/z* (%):

278 (2, $[M^+-H_2O]$), 137 (5, $[C_{10}H_{18}]^+$), 123 (40, $[M-CH_2]^+$), 109 (15, $[M-CH_2]^+$), 95 (36, $[M-CH_2]^+$), 81 (38, $[M-CH_2]^+$), 71 (100, $(CH_3)_2CHCH_2CH_2^+$), 57 (62, $(CH_3)_2CHCH_2^+$).

^{1}H-NMR ($CDCl_3$, 399.65 MHz) δ:

0.82 (12H, H-16, H-17, H-18, H-19, methyls), 0.95-1.61 (19H, m,methyls and methyline protons), 1.68 (3H, s, H-20), 2.00 (2H, t, *J*= 7.6 Hz, H-4), 4.15 (2H, d, *J*= 7.1 Hz, H-1), 5.42 (1H, t, *J*=7.1 Hz, H-2).

^{13}C-NMR ($CDCl_3$, 100.40 MHz) δ:

59.4 (C-1), 123.1 (C-2), 140.3 (C-3), 39.9 (C-4), 25.1 (C-5), 36.6 (C-6), 32.8 (C-7), 37.3 (C-8), 24.5 (C-9), 37.4 (C-10), 32.7 (C-11), 37.3 (C-12), 24.8 (C-13), 39.4 (C-14), 28.0 (C-15), 22.6 (C-16), 22.7 (C-17), 19.7 (C-18), 19.7 (C-19), 16.2 (C-20).

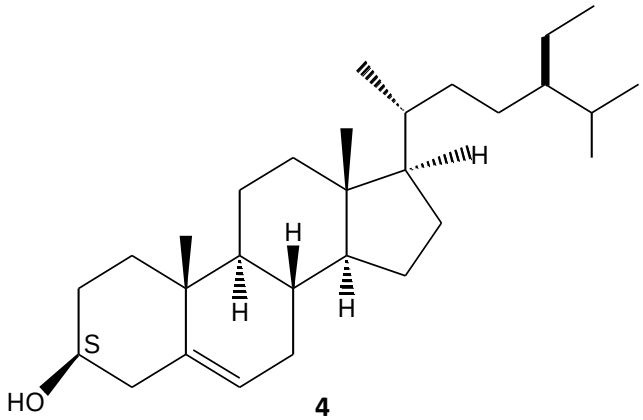
Compound **3** was obtained as a yellow viscous oil and present as the major component (41.2 mg; Figure 3.4) in this plant. It was identified as phytol from GC-MS

analysis (NIST 05 mass-spectral library, 2002) and comparison with reported data (Ming *et al*., 2004), consistent with the molecular formula of $C_{20}H_{40}O$.

The fragmentation of mass spectrum of phytol (**3**) has been discussed in section 4.8.1. Appendix F, F1, F2 and F3 show the mass spectrum, ^{1}H-NMR spectrum, ^{13}C-NMR spectrum and DEPT spectrum of phytol (**3**), respectively which confirmed the presence of phytol (**3**) by comparison with published data (Ming *et al*., 2004).

In addition, the structure of phytol (**3**) suggested that it might be one of the constituents that contributed to the antioxidant activity (section 4.2.2-4.2.4) of the ethyl acetate extract of *P. bleo* as an electron on oxygen atom may be donated in the assay involving electron transfer.

(iv) Structural determination of ß-sitosterol (4)



4

The mass spectrum and the NMR spectral data obtained for compound **4** are shown as follows:

EI-MS *m/z* (%):

414 (100, $[M]^+$), 396 (57, $[M-H_2O]^+$), 381 (43, $[M-H_2O-CH_3]^+$), 354 (8), 329 (42), 303 (38), 273 (20, $[M-C_{10}H_{21}]^+$), 255 (27, $[M-C_{10}H_{23}]^+$), 231 (16), 213 (29), 159 (28), 145 (40), 133 (28), 119 (26), 105 (36), 95 (29), 81 (29), 69 (20), 55 (38).

^{1}H-NMR ($CDCl_3$, 399.65 MHz) δ:

0.69 (3H, s, H-18), 0.82 (3H, s, H-27), 0.83 (3H, s, H-26), 0.85 (3H, s, H-29), 0.94 (3H, br, H-19), 1.02 (3H, s, H-21), 0.97-2.28 (m, CH_2, CH), 3.54 (H-3), 5.36 (br, H-6).

^{13}C-NMR ($CDCl_3$, 100.40 MHz) δ:

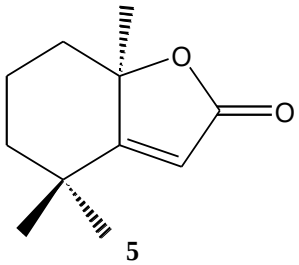
37.2 (C-1), 31.7 (C-2), 71.8 (C-3), 42.3 (C-4), 140.7 (C-5), 121.7 (C-6), 31.9 (C-7), 32.2 (C-8), 50.1 (C-9), 36.5 (C-10), 21.1 (C-11), 39.8 (C-12), 42.3 (C-13), 56.8 (C-14), 24.3 (C-15), 28.2 (C-16), 56.0 (C-17), 11.8 (C-18), 19.4 (C-19), 36.1 (C-20), 18.8 (C-21), 33.9 (C-22), 26.0 (C-23), 45.8 (C-24), 29.1 (C-25), 19.8 (C-26), 19.2 (C-27), 23.1 (C-28), 12.0 (C-29).

Compound **4** was obtained as white coloured needles, identified as ß-sitosterol through GC-MS analysis (NIST 05 mass-spectral library, 2002) and comparison with reported data (Nes *et al.*, 1992). It showed one dark blue spot on the TLC plate when viewed under the short wave UV light. The mass spectrum (Appendix G) showed a molecular ion peak at *m/z* 414 corresponding to the molecular formula $C_{29}H_{50}O$. The NMR spectral data (Appendix G1 and G2) was also consistent with ß-sitosterol by comparison with that reported in the literature (Nes *et al.*, 1992). Sitosterol was also found to be present in *Pereskia aculeata* (Tan *et al*., 2005; Thomas *et al*., 1987).

In the mass spectrum (Appendix G), the peak at *m/z* 329, *m/z* 303 and *m/z* 273 are characteristic peak fragmentations of ring B of ß-sitosterol (**4**) which contains 5-monosubstituted nucleus. The scission of C_{17}-C_{20} in the side chain gave the peak at *m/z* 273. Loss of a water molecule gave a characteristic peak at *m/z* 396, which was followed by the loss of the methyl group to give a peak at *m/z* 381. Besides that, the loss of a water and side chain results in the peak of *m/z* 255.

Sterols are important constituents of all eukaryotes and play a vital role in plant cell membranes. Plant sterols possess valuable physiological activities; they are biogenetic precursors of many hormones and oviposition stimulants of some insects (Harborne, 2001). Sitosterol was reported to poses anti-hyperlipoproteinaemic, antibacterial, antimicotic (Yasukawa *et al*., 1991) and antioxidant activities (Rensburg *et al.*, 2000). Therefore, the presence of ß-sitosterol (**4**) in *P. bleo* might contribute to the antioxidant (section 4.2.2-4.2.4) and antimicrobial activity (section 4.3) of the ethyl acetate extract of *P. bleo*.

(v) Structural determination of dihydroactinidiolide (5)



5

The EI-MS spectrum of compound **5** gave the following mass spectral fragmentations:

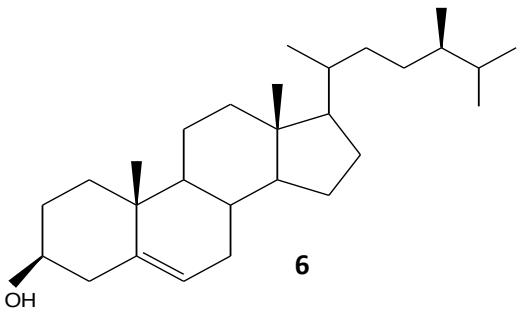
EI-MS *m/z* (%):

180 (15, $[M]^+$), 137 (8), 111 (100), 109 (44), 67 (30).

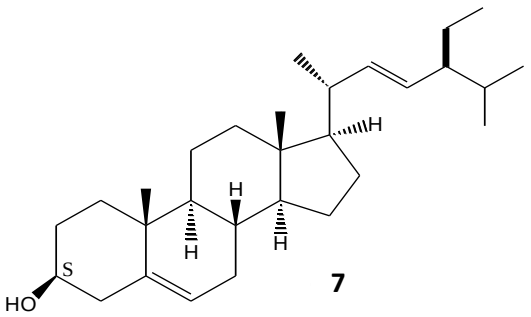
Compound **5** was obtained as red viscous oil and identified as dihydroactinidiolide by GC-MS analysis (NIST 05 mass-spectral library, 2002) and the reported spectroscopic data (Eidman and MacDougall, 2006; Borse *et al*., 2002; Huang *et al.*, 2006). The mass spectrum gave a molecular ion peak at *m/z* 180, consistent with the molecular formula $C_{11}H_{16}O_2$ (Appendix H).

Dihydroactinidiolide (**5**), which is a metabolite of ß-carotene (Sakan *et al.*, 1967), was isolated from the ethyl acetate extract of *P. bleo*. It is a monoterpenoid and structurally similar to the C11-terpene lactones that arise from the biological or oxidative degradation of carotenoids and has been isolated from various plants and insect sources. It has also been identified as the flavor molecule in tea and tobacco (Eidman *et al*., 2006; Borse *et al.*, 2002; Huang *et al*., 2006). To our knowledge, there is no cytotoxcity data of dihydroactinidiolide (**5**) reported in the literature.

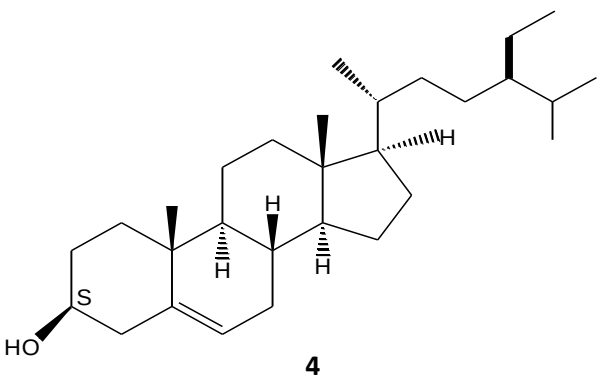
(vi) Structural determination of mixture of sterols (mixture A)



6



7



4

The mixture **A** appeared as white colored needles, identified as consisting of campesterol (**6**, 14.33 %), stigmasterol (**7**, 4.95 %) and β-sitosterol (**4**, 70.21 %) through GC-MS analysis (Appendix I). The sitosterol and stigmasterol were also found to be present in *Pereskia aculeata* (Tan *et al*., 2005; Thomas *et al*., 1987).

Plant sterols are compounds with similar structural skeleton as cholesterol (Berger *et al*., 2004) and found in significant amounts in various parts of plants including seeds, nuts and vegetable oils (Moghadasian, 2000). The most abundant plant sterols are sitosterol, campesterol and stigmasterol (Moreau *et al*., 2002). Plant sterols have been investigated as one of the safe potential alternative methods in lowering plasma cholesterol levels. Several human studies have shown that plant sterols/stanols significantly reduce plasma total and LDL cholesterol (Moghadasian, 2000).

In addition to their cholesterol lowering effect, plant sterols may possess anti-atherosclerosis (Moghadasian *et al*., 1997; Moghadasian *et al*., 1999), antibacterial (Yasukawa *et al*., 1991), anti-inflammation (Bouic, 2001) and antioxidation activities (Rensburg *et al.*, 2000). Particularly, ß-sitosterol (**4**) was reported to poses anti-hyperlipoproteinaemic, antibacterial, antimicotic (Yasukawa *et al*., 1991) and antioxidant activity (Rensburg *et al.*, 2000; Homma *et al*., 2003; Wang *et al*., 2002).

Therefore, the presence of a mixture of sterols (mixture **A**) in *P. bleo* might contribute to the antioxidant (section 4.2.2-4.2.4) and antimicrobial activity (section 4.3) of the ethyl acetate extract of *P. bleo*. The mass-spectral data of campesterol (**6**), stigmasterol (**7**) and β-sitosterol (**4**) are follows.

EI-MS *m/z* (%) (Campesterol, **6**):

400 (42, [M^+]), 382 (34), 367 (20), 315 (30), 289 (30), 55 (100).

The mass spectrum gave a molecular ion peak at *m/z* 400, consistent with the molecular formula to be $C_{28}H_{48}O$ (Appendix I1). It was identified as campesterol through GC-MS analysis (NIST 05 MS Library, 2002) and reported data (Gutierrez, 2006).

EI-MS *m/z* (%) (Stigmasterol, **7**):

412 (16, $[M^+]$), 394 (4, $[M\text{-}H_2O]^+$), 369 (2), 351 (6), 300 (16), 271 (16), 255 (22), 229 (5), 213 (22), 159 (52), 145 (54), 133 (58), 105 (58), 81 (82), 64 (69), 55 (100).

The mass spectrum (Appendix I2) showed the molecular ion peak at *m/z* 412, which corresponding to the molecular formula to be $C_{29}H_{48}O$. The comparison of mass spectral data with NIST mass-spectral library (NIST 05 MS Library, 2002) and reported data (Gutierrez, 2006) confirmed this compound as stigmasterol.

EI-MS *m/z* (%) (ß-Sitosterol, **4**):

414 (84, $[M]^+$), 396 (66, $[M\text{-}H_2O]^+$), 381 (40, $[M\text{-}H_2O\text{-}CH_3]^+$), 354 (10), 329 (48), 303 (49), 273 (30, $[M\text{-}C_{10}H_{21}]^+$), 255 (48, $[M\text{-}C_{10}H_{23}]^+$), 231 (28), 213 (62), 159 (66), 145 (100), 133 (66), 119 (60), 105 (84), 95 (58), 81 (60), 69 (40), 55 (52).

The mass spectrum (Appendix I3) showed a molecular ion peak at *m/z* 414, consistent with the molecular formula $C_{29}H_{50}O$. A comparison of its mass spectral data with NIST mass-spectral library (NIST 05 MS Library, 2002) and published data (Nes *et al.*, 1992) confirmed this compound as ß-sitosterol.

4.8.4 Chemical constituents from the bioactive ethyl acetate extract of *P. grandifolia*

On repeated chromatographic purification of the active ethyl acetate fraction, compound **1** was identified as 2,4-di-tert-butylphenol, **2** as α-tocopherol, **4** as ß-sitosterol, **8** as phytone and the mixture **B** was consisted of methyl palmitate (**9**), methyl oleate (**10**), methyl stearate (**11**) and 2,4-di-tert-butylphenol (**1**). All the compounds have not been reported for this plant. The structures of compounds **1**, **2**, **4**, **8**, **9**, **10** and **11** are illustrated in Figure 4.41. It is interesting to note that 2,4-ditert-butylphenol (**1**), α-tocopherol (**2**) and ß-sitosterol (**4**) were also isolated from *P. bleo* as previously reported (section 4.8.1).

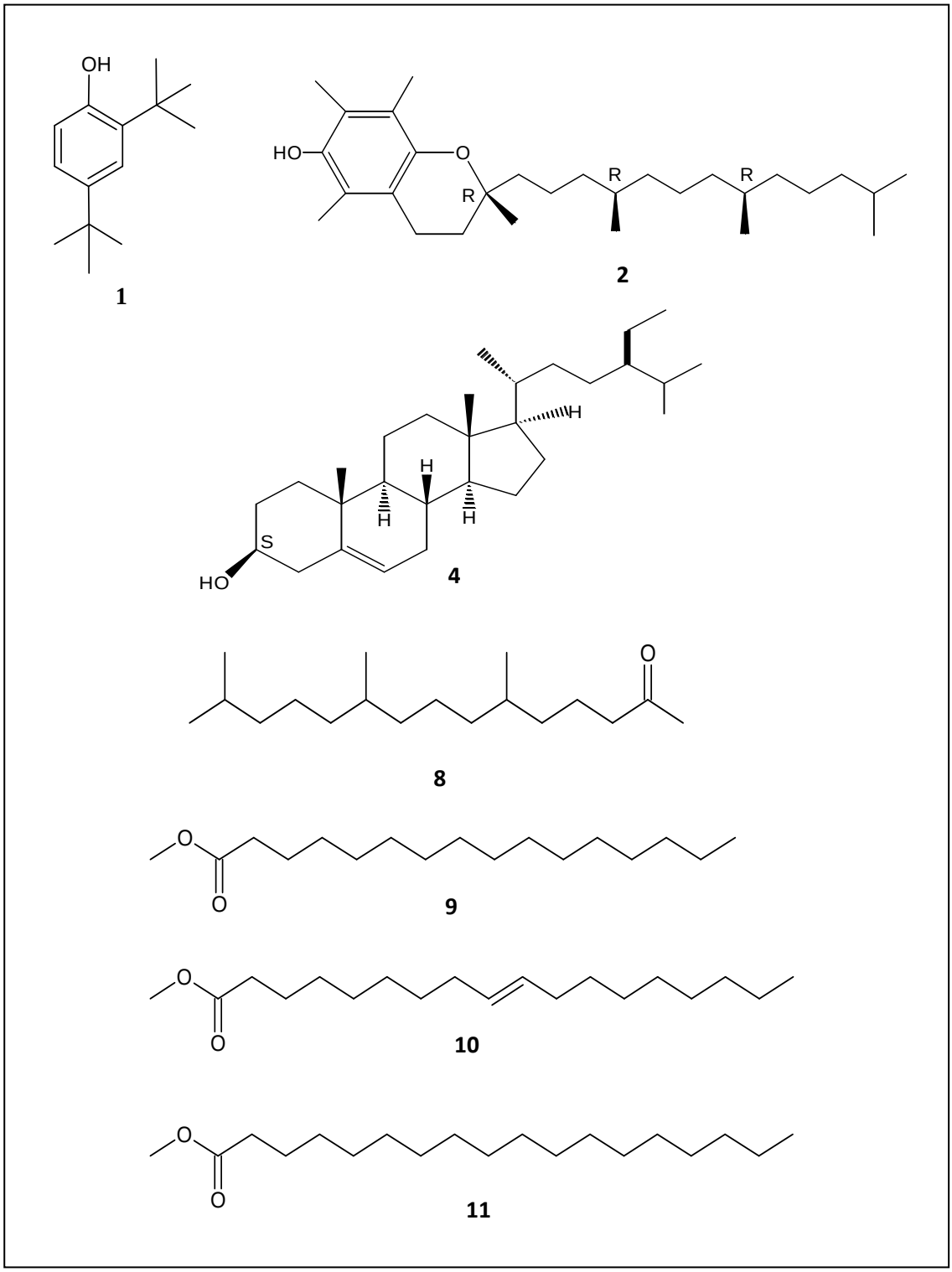
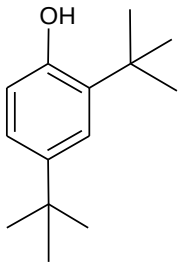


Figure 4.41: Chemical constituents from the bioactive ethyl acetate extract of *P. grandifolia*

(i) Structural determination of 2,4-di-tert-butylphenol (1)



1

Compound **1** was identified as 2,4-di-tert-butylphenol by GC-MS analysis which gave its mass-spectral data as follows:

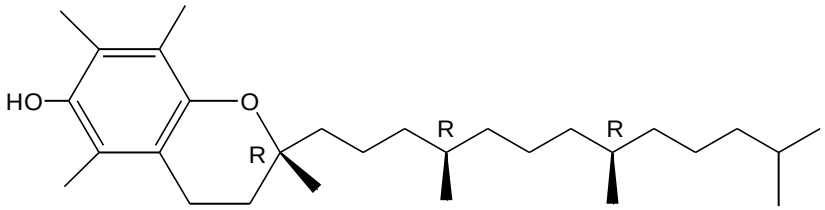
EI-MS *m/z* (%):

206 (15, $[M]^+$), 191 (100, $[M- CH_3]^+$), 57 (32).

Compound **1** was obtained as yellow coloured crystalline powder. The mass spectrum gave the molecular ion peak at *m/z* 206, which suggested a molecular formula of $C_{14}H_{22}O$ (Appendix J). The spectrum showed that a methyl group is lost readily at *m/z* 191. The mass-spectral data was also consistent with the published data (Kamitori *et al.*, 1984).

Based on previous reports (Yoshioka *et al*., 1995; Yu *et al*., 1997; Tanaka *et al*., 1997; Schreck *et al*, (1992; Mitova *et al*., 2003; Russo *et al*., 2009; Kozubek and Tyman, 1999), 2,4-di-tert-butylphenol (**1**) might be responsible for the reducing capacity (section 4.2.1), antioxidant activity (section 4.2.2-4.2.4), antimicrobial activity (section 4.3) and cytotoxic activity (section 4.4) of the ethyl acetate extract of *P. grandifolia*.

(ii) Structural determination of α-tocopherol (2)



2

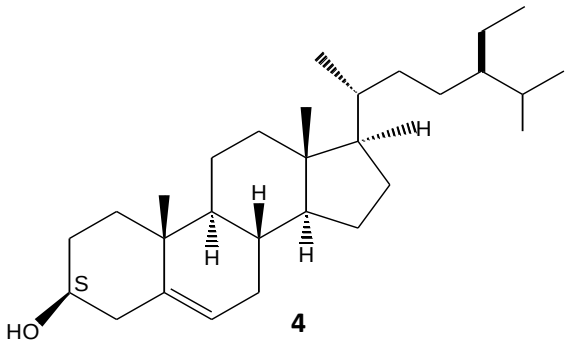
Compound 2 obtained as yellow vicous oil was identified as α-tocopherol by GC-MS which gave the mass-spectral fragmentation as follows:

EI-MS *m/z* (%):

430 (20, [M^+]), 165 (100, $C_{10}H_{13}O_2$), 136 (10), 121 (12), 57 (42).

The mass spectrum gave a molecular ion peak at *m/z* 430, which was consistent with the molecular formula $C_{29}H_{50}O_2$ (Appendix K). Based on previous reports (Bermudez *et al*., 2007; Choi and Lee, 2009; Lamson and Brignall, 2003; Drisko *et al*, 2003; Bektic *et al*., 2005; Kirsh *et al*., 2006; Kampa *et al*., 2000; Zang *et al*., 2000), α-tocopherol (**2**) which is a phenolic compound might be one of the constituent responsible for the reducing capacity (section 4.2.1), antioxidant activity (section 4.2.2-4.2.4), and cytotoxic activity (section 4.4) of the ethyl acetate extracts of *P. grandifolia*.

(iii) Structural determination of ß-sitosterol (4)



4

The EI-MS spectrum of compound **4** gave the following mass spectral fragmentations:

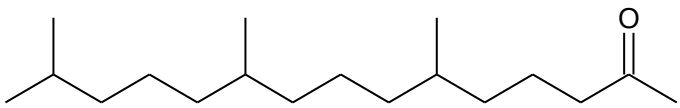
EI-MS *m/z* (%):

414 (100, $[M]^+$), 396 (80, $[M\text{-}H_2O]^+$), 381 (40, $[M\text{-}H_2O\text{-}CH_3]^+$), 354 (8), 329 (44), 303 (40), 273 (20, $[M\text{-}C_{10}H_{21}]^+$), 255 (30, $[M\text{-}C_{10}H_{23}]^+$), 231 (14), 213 (34), 159 (34), 145 (54), 133 (34), 119 (32), 105 (46), 81 (38), 69 (24), 55 (48).

The mass spectrum (Appendix L) showed a molecular ion peak at *m/z* 414 corresponding to the molecular formula $C_{29}H_{50}O$. The comparison of its mass spectral data with NIST mass-spectral library (NIST 05 MS Library, 2002) and published data (Nes *et al.*, 1992) confirmed this compound as ß-sitosterol.

According to previous reports (Moghadasian *et al*., 1997; Moghadasian *et al*., 1999; Yasukawa *et al*., 1991; Bouic, 2001; Rensburg *et al.*, 2000; Homma *et al*., 2003; Wang *et al*., 2002), the presence of ß-sitosterol (**4**) might contribute to the antioxidant (section 4.2.2-4.2.4) and antimicrobial activity (section 4.3) of the ethyl acetate extract of *P. grandifolia.*

(iv) Structural determination of phytone (8)



8

The EI-MS spectrum of compound **8** gave the following mass spectral fragmentations:

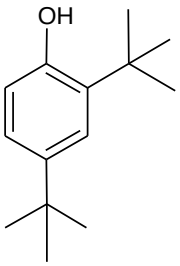
EI-MS *m/z* (%):

268 (1, $[M]^+$), 250 (3, $[M\text{-}OH_2]^+$), 235 (1), 225 (1), 210 (2), 194 (2), 179 (3), 165 (4), 151 (2), 137 (5), 109 (22), 95 (20), 85 (25), 71 (44), 58 (100).

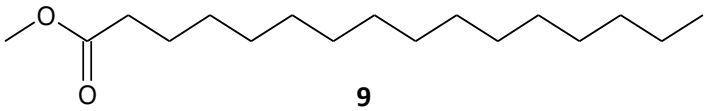
Compound **8** was obtained as yellow coloured crystalline powder. The mass spectrum gave the molecular ion peak at *m/z* 268, which suggested the molecular formula of $C_{18}H_{36}O$ (Appendix M). The comparison with NIST mass-spectral library (NIST 05 MS Library, 2002) and published data (Uechi *et al*., 2007) confirmed this compound as phytone.

Phytone (**8**) is a phytol degradation biomarker in chlorophyll (Alves *et al*., 2001; Figueiredo *et al*., 2007) and was patented in United States in 1994 (Patent Number: 5,318.783) as it showed biological activity against herbivorous insects, especially insect graminivores.

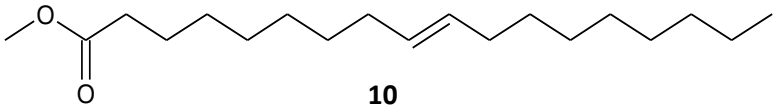
(v) Structural determination of mixture B



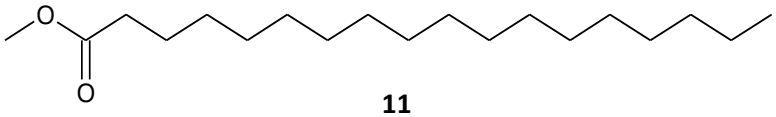
1



9



10



11

The mixture **B** appeared as yellow vicious oil and identified as consisting of 2,4-di-tert-butylphenol (**1,** 80.82 %), methyl palmitate (**9,** 15.81 %), methyl oleate (**10,** 1.74 %) and methyl stearate (**11,** 1.63 %) by GC-MS analysis (Appendix N).

The presence of 2,4-di-tert-butylphenol (**1**) in the mixture was identified by GC-MS which gave the full mass spectral data (Appendix N1). Comparison with published data (Kamitori *et al.*, 1984) also confirmed this identification.

EI-MS *m/z* (%) (2,4-di-tert-butylphenol, **1**):

206 (16, $[M]^+$), 191 (100, $[M\text{-}CH_3]^+$), 175 (4), 163 (5), 147 (2), 141 (1), 135 (2), 128 (2), 121 (2), 115 (4), 107 (4), 91 (4), 74 (4), 65 (1), 57 (14), 51 (1).

The mass spectrum gave the molecular ion peak at *m/z* 206, which suggested a molecular formula of $C_{14}H_{22}O$ (Appendix N1). A base peak at *m/z* 191 showed the loss of a methyl group.

The EI-MS spectrum of compound **9** gave the following mass spectral fragmentations:

EI-MS *m/z* (%) (methyl palmitate, **9**):

270 (6, $[M]^+$), 239 (4, $[M- OCH_3]^+$, $C_{16}H_{31}O_1^+$), 227 (10), 213 (2), 199 (4), 185 (5), 171 (5), 157 (2), 143 (22), 129 (8), 115 (4), 107 (1), 97 (7), 87 (70, $CH_2CH_2CO_2CH_3^+$), 74 (100, $C_3H_6O_2^+$), 65 (1), 55 (30).

The mass spectrum gave the molecular ion peak at *m/z* 270, which suggested a molecular formula to be $C_{17}H_{34}O_2$ (Appendix N2). A comparison with NIST mass-spectral library (NIST 05 MS Library, 2002) and Adams (2001) confirmed this compound as methyl palmitate (or methyl hexadecanoate).

The EI-MS spectrum of compound **10** gave the following mass spectral fragmentations:

EI-MS *m/z* (%) (Methyl oleate, **10**):

296 (2, $[M]^+$), 264 (12), 246 (1), 235 (2), 222 (8), 207 (2), 194 (2), 180 (8), 166 (4), 152 (6), 137 (10), 123 (14), 111 (20), 97 (39), 83 (42), 69 (56), 55 (100).

The mass spectrum gave the molecular ion peak at *m/z* 296, which suggested amolecular formula to be $C_{19}H_{36}O_2$ (Appendix N3). A comparison with NIST mass-spectral library (NIST 05 MS Library, 2002) and Adams (2001) confirmed this compound as methyl oleate.

The EI-MS spectrum of compound **11** gave the following mass spectral fragmentations:

EI-MS *m/z* (%) (Methyl stearate, **11**):

298 (6, $[M]^+$), 267 (2), 255 (8), 241 (1), 227 (1), 213 (2), 199 (6), 185 (4), 143 (21), 129 (6), 111 (3), 97 (8), 87 (69, $CH_2CH_2CO_2CH_3^+$), 74 (100, $C_3H_6O_2^+$), 55 (30).

The mass spectrum gave the molecular ion peak at *m/z* 298, which suggested the molecular formula to be $C_{19}H_{38}O_2$ (Appendix N4). A comparison with NIST mass-spectral library (NIST 05 MS Library, 2002) and Adams (2001) confirmed this compound as methyl stearate (methyl octadeacanoate).

4.9 Cytotoxic and apoptosis effects of chemical constituents isolated from the bioactive ethyl acetate extracts of *P. bleo* and *P. grandifolia*

It is generally known that ethnomedical data substantially increases the chances of finding active plants relative to a random approach (Cordell *et al*., 1991; Chapuis *et al*., 1988; Cragg *et al*., 1994). The consequence is that, once having found activity in the plant from the ethopharmacological observation (e.g. raw or concoction brewed from the plant leaves shows effect for cancer treatment), there is a desire to determine the chemical structures of the chemical constituents that are responsible for the activity, as not all the chemical constituents in the extracts have the same activity.

In the present study, the chemical constituents isolated from the bioactive extracts of *P. bleo* and *P. grandifolia* were tested for their cytotoxic activity against five human carcinoma cell lines, namely KB, CasKi, HCT 116, MCF7 and A549, and non-cancer cell line (MRC-5). Only chemical constituents which were active in cytotoxic activity and detected with DNA fragmentation were tested in the gene expression study. According to US NCI plant screening program, a pure compound is generally considered to have active cytotoxic effect if the IC_{50} value, following incubation between 48 to 72 hs is ≤ 4 μg/ml (Lee and Houghton, 2005; Boik, 2001; Geran *et al*., 1997; Geran *et al*., 1972; Swanson and Pezzuto, 1990). In the current study, the chemical constituents that showed cytotoxic effect with IC_{50} value ≤ 6 μg/ml were subjected to apoptosis detection and apoptotic-related gene expression study.

For the gene expression study, concentration of the chemical constituent needed to achieve 50 % growth inhibition (IC_{50}) was used to stimulate the cells over the period of 6 h. The mRNA expression levels of the apoptotic-related genes, i.e. p53, caspase-3 and c-myc in treated cells were carried out using the two-step LUX RT-qPCR assay as described in section 3.7.3. The mRNA level of ß-actin was used as internal reference gene for template levels.

4.9.1 Cytotoxic and apoptosis effects of 2,4-di-tert-butylphenol (1)

(i) Cytotoxic activity of 2,4-di-tert-butylphenol (1)

Antioxidant phenols have been epidemiologically associated with a reduced incidence of cancer (Willet *et al*., 1995; Trichopoulou, 1995), while several *in vitro* and *in vivo* studies have indicated that they exhibit anticancer activity *via* different molecular pathways (Liang *et al*., 1999; Ahn *et al*., 1999). Phenols such as rutin and syringic acid were reported to have chemopreventive activity in laboratory animal models through modulation of cell proliferation (Tanaka *et al*., 1999) and antiproliferative activity towards HL-60 cells (Hirota *et al*., 2000), respectively.

2,4-Di-tert-butylphenol **(1)** which is a phenolic compound, was isolated from *P. bleo* as well as *P. grandfolia*. In the present study, 2,4-di-tert-butylphenol (**1**) displayed very remarkable cytotoxic activity against KB cells with an IC_{50} value of 0.81 µg/ml and strong cytotoxicity against MCF7 (IC_{50} 5.75 µg/ml), A549 (IC_{50} 6 µg/ml) and CasKi cells (IC_{50} 4.5 µg/ml). The results of cytotoxicity screening (IC_{50} values in µg/ml and µM) of 2,4-di-tert-butylphenol (**1**) are summarized in Table 4.20. The *in vitro* growth inhibitions of 2,4-di-tert-butylphenol (**1**) against selected human cell lines are shown in Figure 4.42. Percentage of inhibition was increased with increment in 2,4-

di-tert-butylphenol (**1**) concentrations. The above data thus support the findings that phenolic antioxidants exert cytoctoxic activity against cancer cells (Russo *et al*., 2009; Dedoussis *et al*., 2005; Kozubek and Tyman, 1999).

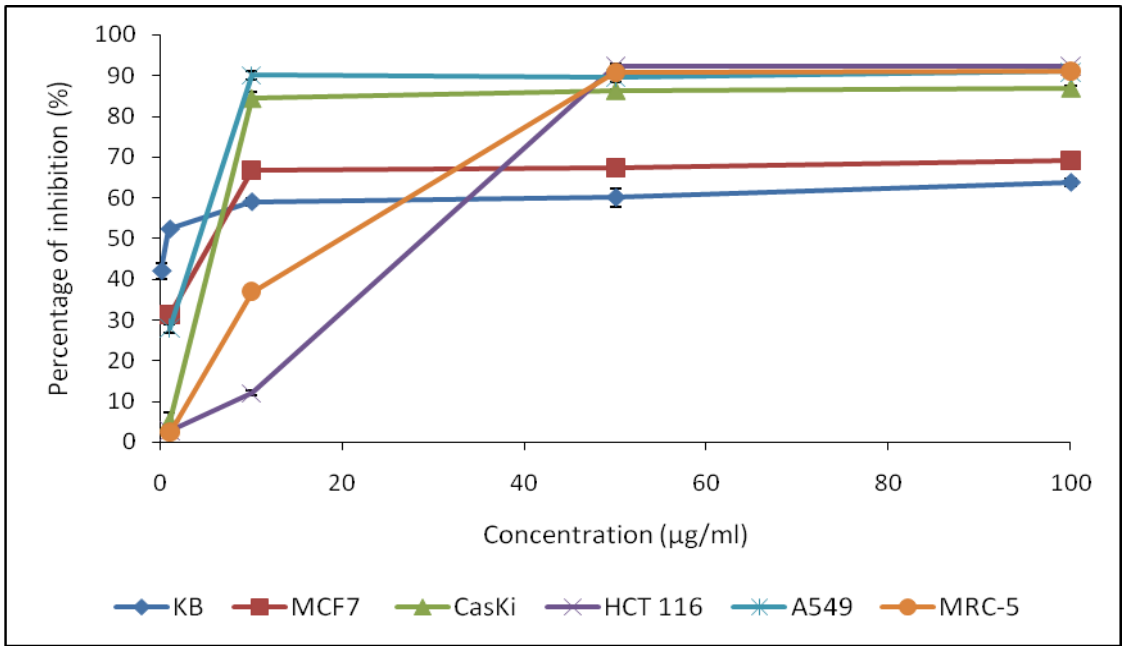


Figure 4.42: The *in vitro* growth inhibitions of 2,4-di-tert-butylphenol (1) against selected human cell lines determined by neutral red cytotoxicity assay. Each value is expressed as mean ± standard deviation of three measurements.

Table 4.20: Cytotoxic activity (IC_{50} values) of 2,4-di-tert-butylphenol (1) against selected human cell lines

Compound	Cytotoxicity (IC_{50}) in µg/ml (µM)					
	KB	MCF7	CasKi	HCT 116	A549	MRC-5
2,4-di-tert-butylphenol (**1**)	**0.81** (3.93)	**5.75** (27.91)	**4.5** (21.84)	29 (140.78)	**6** (29.13)	20 (97.09)
Doxorubicin[a]	$1.3x10^{-2}$ (0.023)	$7.6x10^{-2}$ (0.139)	$6.0x10^{-3}$ (0.011)	$3.6x10^{-1}$ (0.663)	$2.2x10^{-1}$ (0.401)	$5.5x10^{-1}$ (1.01)

[a] Doxorubicin was used as the reference compound

(ii) Induction of apoptosis by 2,4-di-tert-butylphenol (1) on selected cells

Phenolic compounds such as rutin have been reported to induce apoptosis in various cancer cell lines (Romero *et al*., 2002). Gallic acid has also been shown to induce apoptosis in lung cancer cells (Ohno *et al*., 2001), human stomach cancer cells, colon adenocarcinoma cells (Yoshioka *et al.*, 2000) and human erythroleukemic cells (Aoki *et al*., 2001). Furthermore, the phenolic antioxidant BHA which is structurally related to 2,4-di-tert-butylphenol (**1**), was reported to induce mitochondria-cytochrome c release-caspase activation pathway leading to apoptosis (Kong *et al*., 2001).

In DeadEnd^{TM} Colorimetric Apoptosis Detection System (Promega), 2,4-di-tert-butylphenol (**1**) was able to induce apoptosis in KB, MCF7, A549 and CasKi cell lines as indicated by the darker staining of nuclei of treated cells. The DNA fragmentation was evident after 24 h treatment. The exposure to the vehicle (DMSO; negative control) did not cause nuclear alterations as confirmed by the absence of staining. The results thus support the findings that phenolic antioxidants induce apoptosis in cancer cells (Dedoussis *et al*., 2005).

(iii) Expression level of apoptosis-related genes in 2,4-di-tert-butylphenol (1)-treated cells

KB cell line

Figure 4.43 shows the time dependency effects of mRNA levels of p53, caspase-3 and c-myc in KB cells induced 2,4-di-tert-butylphenol (**1**). The results strongly indicate that 2,4-di-tert-butylphenol (**1**) killed the KB cells through apoptosis mechanism *via* the activation of p53 and caspase-3. The KB cells treated with 2,4-di-tert-butylphenol (**1**) showed significantly strong up-regulation in p53 and caspase-3 expression. Figure 4.43 shows that the steady state mRNA levels of p53 and caspase-3

were increased drastically when the KB cells were treated with the extract at 15 min (99.9- and 145.9-folds increase as compared to control cells, respectively) and reduced to 45.2- and 7.6-fold, respectively, after 1 h incubation, increased again to 93.8- and 101.9-fold, respectively, at 3 h and finally 227.6- and 31.8-fold, respectively, at 6 h.

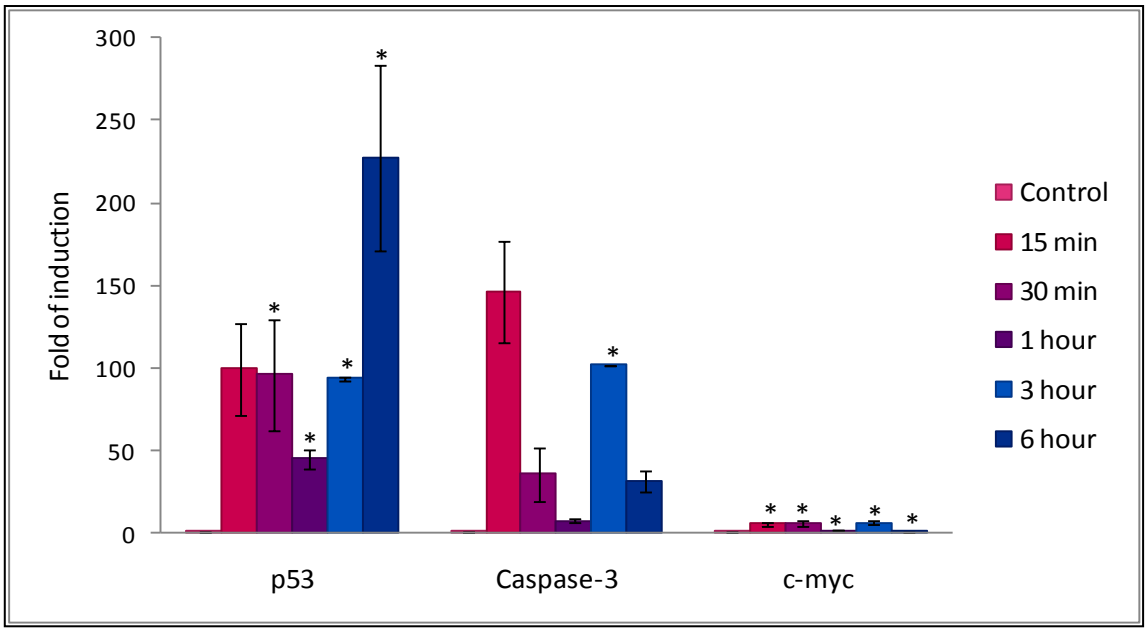


Figure 4.43: The mRNA expression of p53, caspase-3 and c-myc detected in KB cells treated with 2,4-di-tert-butylphenol (1) for different durations. Results are expressed as means ± standard deviation. *: Significant difference from the control ($p < 0.05$).

MCF7 cell line

Figure 4.44 shows the time dependency effects of p53, caspase-3 and c-myc mRNA levels in MCF7 cells induced with 2,4-di-tert-butylphenol (**1**). Interestingly, the mRNA expression level of p53, caspase-3 and c-myc were highest at 6 h incubation (31-, 12- and 38.2-fold increase as compared to control cells, respectively). In general, an increased level of p53 expression was observed in MCF7 cells treated with 2,4-di-tert-butylphenol (**1**) except a significant drop ($p < 0.05$) at 1 h of incubation. Whilst, a reduction of c-myc expression level was observed in treated MCF7 cells from 30 min to

3 h incubation after a drastic increase at 15 min (9.9-fold increase as compared to control cells). However, the c-myc expression increased again at 6 h (38.2-fold increase as compared to control cells). In summary, the result here strongly indicates that 2,4-di-tert-butylphenol (**1**) killed the MCF7 cells through apoptosis mechanism mainly *via* the activation of p53 and c-myc.

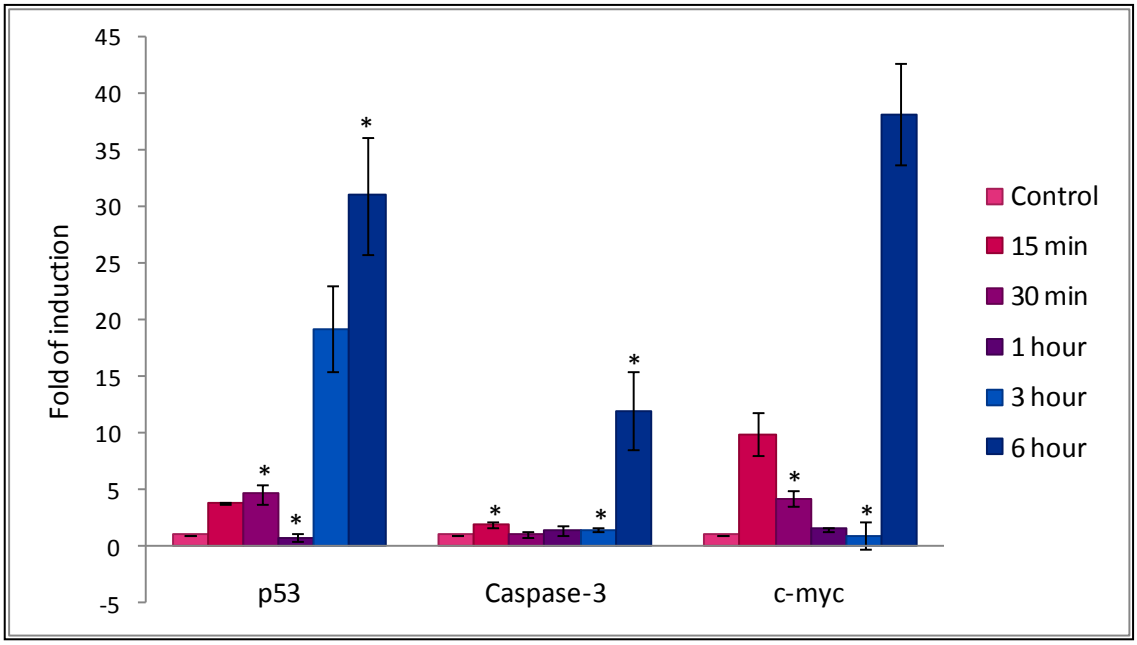


Figure 4.44: The mRNA expression of p53, caspase-3 and c-myc detected in MCF7 cells treated with 2,4-di-tert-butylphenol (1) for different durations. Results are expressed as means ± standard deviation. *: Significant difference from the control ($p < 0.05$).

CasKi cell line

Figure 4.45 shows the time dependency effects of p53, caspase-3 and c-myc mRNA levels in CasKi cells induced with 2,4-di-tert-butylphenol (**1**). It is noteworthy to mention that the trend of p53, caspase-3 and c-myc mRNA expression levels in CasKi cells induced with 2,4-di-tert-butylphenol (**1**) was similar. The mRNA expression levels of p53, caspase-3 and c-myc were lower than the control at incubation time of 15 min to 3 h and increased drastically when the cells were treated with the

extract at 6 h (3.7-, 1.8- and 2.0-folds increase as compared to the control cells, respectively). However, the statistical analysis did not show any significant difference in the expression level of caspase-3 compared to the control. In summary, the results indicate that 2,4-di-tert-butylphenol (**1**) killed the CasKi cells through apoptosis mechanism mainly *via* the activation of p53 while the role of caspase-3 and c-myc cannot be ruled out.

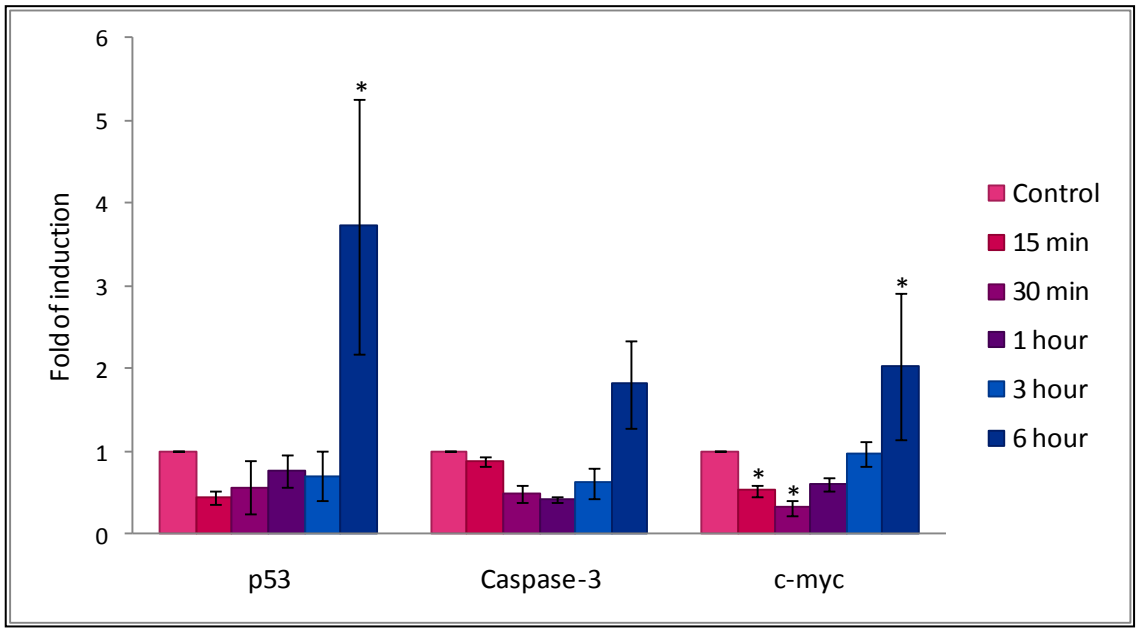


Figure 4.45: The mRNA expression of p53, caspase-3 and c-myc detected in CasKi cells treated with 2,4-di-tert-butylphenol (1) for different durations. Results are expressed as means ± standard deviation. *: Significant difference from the control ($p < 0.05$).

A549 cell line

Figure 4.46 shows the time dependency effects of p53, caspase-3 and c-myc mRNA levels in A549 cells induced with 2,4-di-tert-butylphenol (**1**). The results strongly indicate that 2,4-di-tert-butylphenol (**1**) killed the A549 cells through apoptosis mechanism mainly *via* the activation of p53. There was an increase in the level of p53 expression observed in the A549 cells treated with the increasing of incubation time

with 2,4-di-tert-butylphenol (**1**). Figure 4.46 shows that the steady state mRNA level of p53 was 1.7-fold increased when treated with 2,4-di-tert-butylphenol (**1**) at 15 min. The p53 expression was significantly increased ($p < 0.05$) at 30 min (4.5-fold increase as compared to control cells) and 1 h (4.6-fold increase as compared to control cells), respectively. However, the expression levels of p53 were reduced to 1.7- and 1.1-fold, after 3 h and 6 h respectively. On the contrary, the expression levels of caspase-3 and c-myc were slightly increased to 1.1- and 1.5-fold, respectively, after 15 min incubation and decreasing thereafter to the level below control for the incubation time of 30 min to 3 h before slightly increased again to 1.2- and 1.4-fold, respectively, at 6 h.

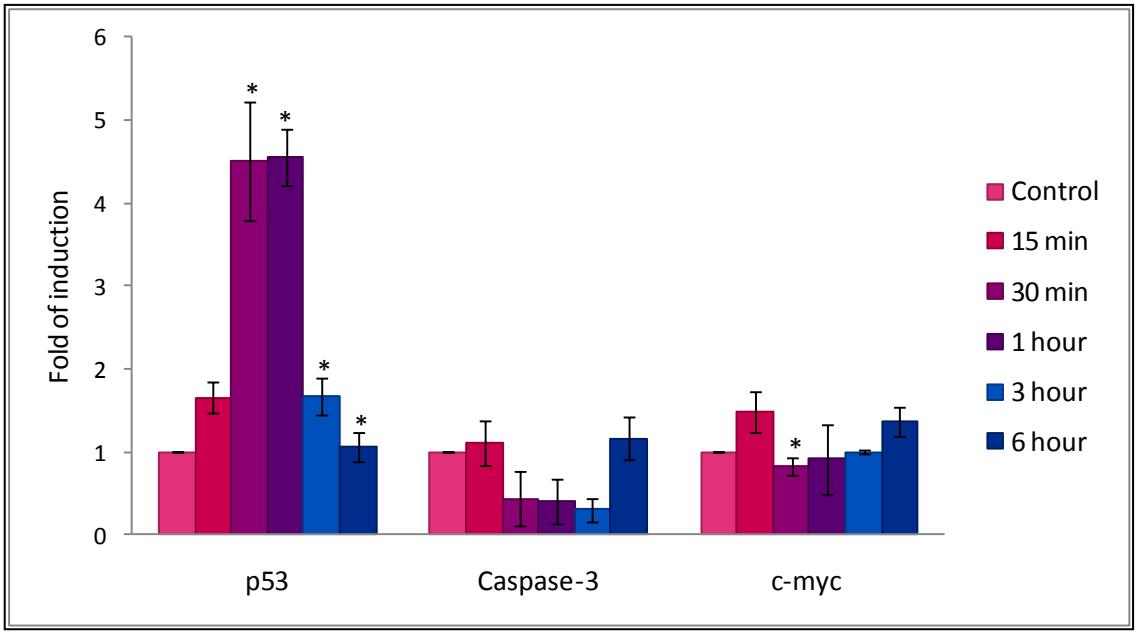


Figure 4.46: The mRNA expression of p53, caspase-3 and c-myc detected in A549 cells treated with 2,4-di-tert-butylphenol (1) for different durations. Results are expressed as means ± standard deviation. *: Significant difference from the control ($p < 0.05$).

4.9.2 Cytotoxic and apoptosis effects of α-tocopherol (2)

(i) Cytotoxic activity of α-tocopherol (2)

α-Tocopherol (**2**) was isolated from *P. bleo* as well as *P. grandfolia*. The results of cytotoxicity screening (IC_{50} values in µg/ml and µM) of α-tocopherol (**2**) are summarized in Table 4.21. The *in vitro* growth inhibitions of α-tocopherol (**2**) against selected human cell lines are shown in Figure 4.47. Percentage of inhibition was increased with the increment in α-tocopherol (**2**) concentrations.

In the present study, α-tocopherol (**2**) displayed pronounced cytotoxicity against CasKi (IC_{50} 6 µg/ml) and A549 (IC_{50} 6 µg/ml) but weak cytotoxicity against HCT 116 cell line (IC_{50} 31 µg/ml). The strong cytotoxicity of α-tocopherol (**2**) against CasKi cells confirms the research findings of Al-Sherbini *et al*. (2009) that α-tocopherol (**2**) was significantly decreased the viability of cervical cancer cells in a concentration dependant manner. Whilst, the weak cytotoxicity of α-tocopherol (**2**) against HCT 116 cell line in the present study support the finding reported by Campbell *et al.* (2006) that α-tocopherol (**2**) is a poor inhibitor against HCT 116 colon cancer cells. In addition, the low cytotoxicty of α-tocopherol (**2**) against MRC 5 cells (IC_{50} 30.5 µg/ml) in the present study also supporta the result reported by Melnikova *et al.* (1999). The other result of obtained here is also consistent with other reports (Hao *et al*., 2009; Bermudez *et al*., 2007; Lamson and Brignall, 1999; Drisko *et al*., 2003; Kogure *et al*., 2005; Wu *et al*., 2009) on cytotoxic activities in human cell lines.

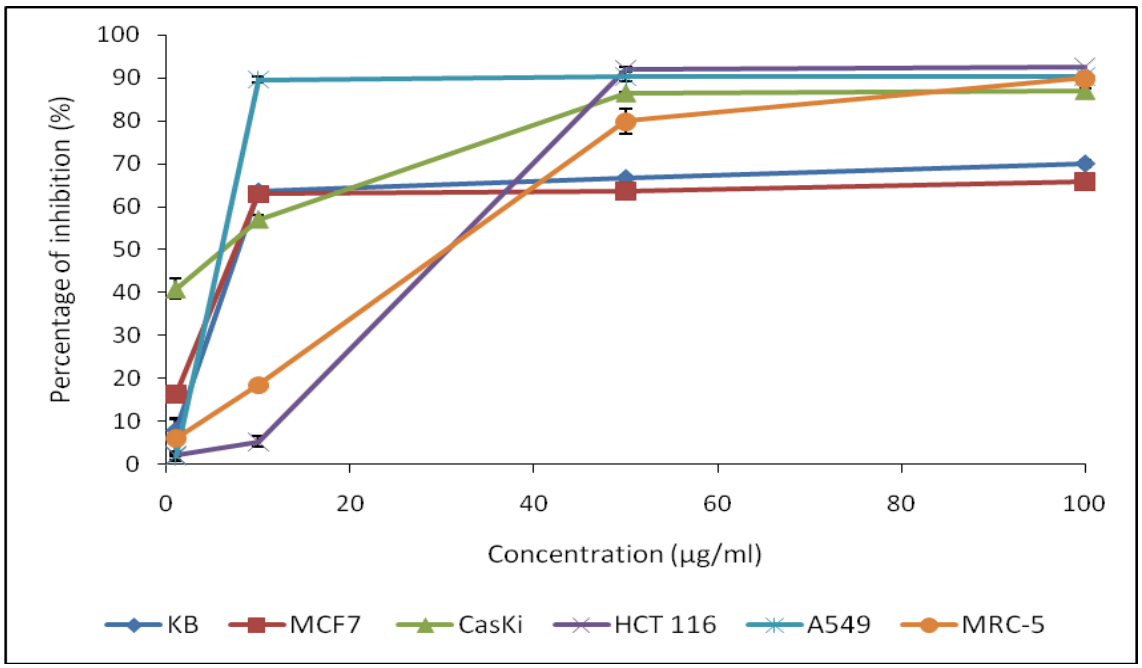


Figure 4.47: The *in vitro* growth inhibitions of α-tocopherol (2) against selected human cell lines determined by neutral red cytotoxicity assay. Each value is expressed as mean ± standard deviation of three measurements.

Table 4.21: Cytotoxic activity (IC_{50} values) of α-tocopherol (2) against selected human cell lines

Compound	Cytotoxicity (IC_{50}) in µg/ml (µM)					
	KB	MCF7	CasKi	HCT 116	A549	MRC-5
α-Tocopherol (**2**)	8 (18.60)	7.5 (17.44)	**6** (13.95)	31 (72.09)	**6** (13.95)	30.5 (70.93)
Doxorubicin[a]	$1.3x10^{-2}$ (0.023)	$7.6x10^{-2}$ (0.139)	$6.0x10^{-3}$ (0.011)	$3.6x10^{-1}$ (0.663)	$2.2x10^{-1}$ (0.401)	$5.5x10^{-1}$ (1.01)

[a] Doxorubicin was used as the reference compound

(ii) Induction of apoptosis by α-tocopherol (2) on selected cells

In order to determine whether the cytotoxic effects of α-tocopherol (**2**) was due to apoptosis, CasKi and A549 cells were treated with α-tocopherol (**2**) for 24 h and modified TUNEL assay (DeadEnd™ Colorimetric Apoptosis Detection System,

Promega) was performed. The nuclei of CasKi and A549 cells treated with α-tocopherol (**2**) were stained dark brown while none of the cell nucleus was stained dark in negative control. The results here thus support the findings that α-tocopherol (**2**) is able to induce apoptosis in cancer cells (Bermudez *et al*., 2007; Schwartz *et al*., 2001; McCormick and Parker, 2004; Melnikova *et al*., 1999; Kline *et al.*, 2001; Boscoboinik *et al*., 1991).

(iii) Expression level of apoptosis-related genes in α-tocopherol (2)-treated cells

CasKi cell line

Figure 4.48 shows the time dependency effects of mRNA levels of p53, caspase-3 and c-myc in CasKi cells induced α-tocopherol (**2**). After incubation with α-tocopherol (**2**) for various duration, p53 expression was strongly up-regulated significantly ($p < 0.05$) whereas caspase-3 and c-myc expression was substantially down-regulated significantly ($p < 0.05$). Figure 4.48 shows that the steady state mRNA level of p53 was significantly increased ($p < 0.05$) when the cells were treated with α-tocopherol (**2**) at 15 min (5.1-fold increase as compared to control cells) and increased significantly again at 30 min (10-fold increase as compared to control cells) at 1 h. In summary, the results strongly indicate that α-tocopherol (**2**) killed the CasKi cells through apoptosis mechanism mainly *via* the activation of p53. The result here thus support the findings that α-tocopherol (**2**) exhibits cancer formation by stimulating the expression of p53 cancer suppressor gene (Schwartz *et al*., 1993; Melnikova *et al*., 1999; Kline *et al.*, 2001).

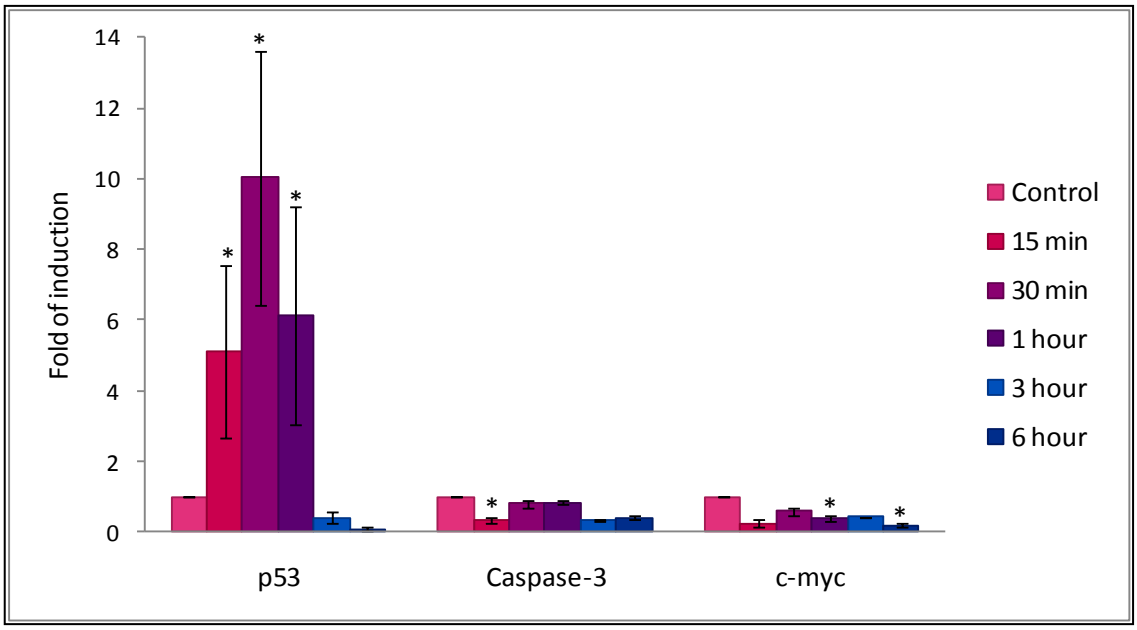


Figure 4.48: The mRNA expression of p53, caspase-3 and c-myc detected in CasKi cells treated with α-tocopherol (2) for different durations. Results are expressed as means ± standard deviation. *: Significant difference from the control ($p < 0.05$).

A549 cell line

Figure 4.49 shows the time dependency effects of mRNA levels of p53, caspase-3 and c-myc in A549 cells induced α-tocopherol (**2**). Figure 4.49 shows that the steady state mRNA level of caspase-3 was increased drastically when the cells were treated with α-tocopherol (**2**) at 15 min (6.9-fold increase as compared to control cells) and decreasing thereafter to 1.2-fold at 6 h. At the meanwhile, the p53 expression was slightly increased significantly ($p < 0.05$) at 15 min (1.3-fold increase as compared to control cells) and decreasing thereafter, to the level below baseline. In addition, a significant increase ($p < 0.05$) was observed in c-myc expression at 15 min (2.7-fold increase as compared to control cells) and decreased to 1.3- and 1.4-fold at 30 min and 1 h, respectively, and decreasing thereafter, to the level below the baseline. Thus, the result here strongly indicates that α-tocopherol (**2**) killed the A549 cells through

apoptosis mechanism mainly *via* the activation of caspase-3 while the role of p53 and c-myc cannot be totally ruled out.

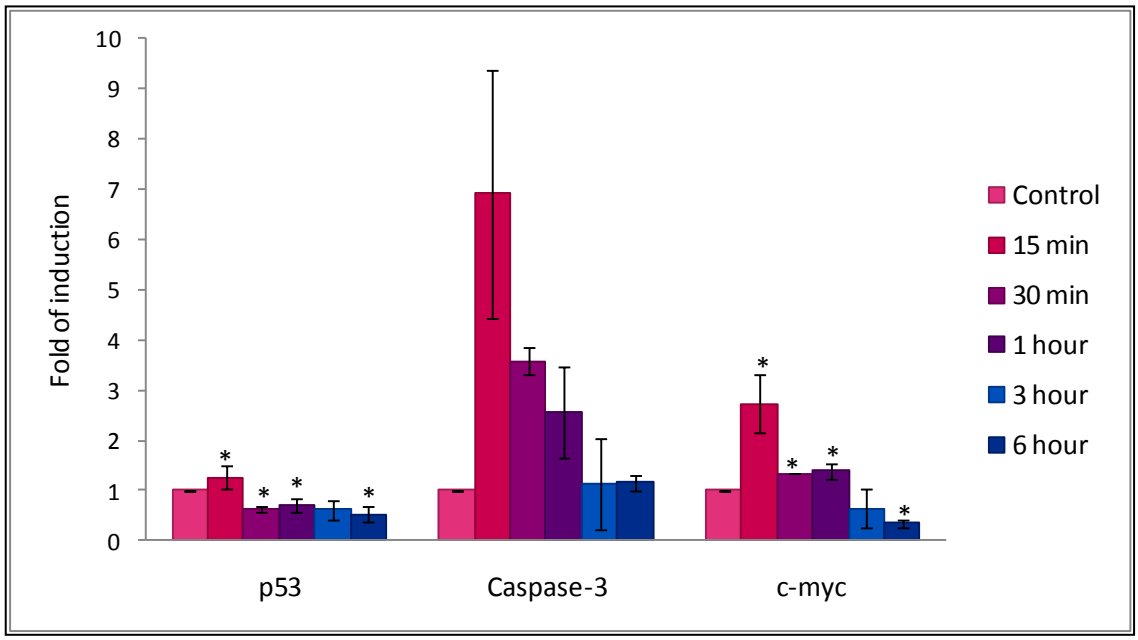


Figure 4.49: The mRNA expression of p53, caspase-3 and c-myc detected in A549 cells treated with α-tocopherol (2) for different durations. Results are expressed as means ± standard deviation. *: Significant difference from the control ($p < 0.05$).

4.9.3 Cytotoxic effect of phytol (3)

In the present study, phytol (**3**) was isolated from the ethyl acetate extract of *P. bleo*. The *in vitro* growth inhibitions of phytol (**3**) against selected human cell lines are shown in Figure 4.50. Percentage of inhibition was increased with the increment in phytol (**3**) concentrations. According to previous study, phytol (**3**) showed some antiproliferative effect against P388 mouse lymphocytic leukemia cells (Phutdhawong *et al.*, 2004), molt 4B lymphoid leukemia cells (Hibasami *et al.*, 2002), HT-29 human colon cancer cells, MG-63 osteosarcoma cells, HeLa human cervix carcinoma cells, HL-60 human promyelocytic leukemia cells and AZ-521 gastric cancer cells (Lee *et al.*,

1999; Block *et al*., 2004). Whilst, phytol (**3**) demonstrated relatively strong cytotoxic activity against KB cells (IC_{50} 7.1 µg/ml) in comparison with other human cell lines in the present study (Table 4.22).

As reported by previous study, phytol (**3**) was found to induce apoptotic cell death (Komiya *et al*, 1999). However, phytol (**3**) was not subjected to apoptosis detection in the present study as only the chemical constituents that showed cytotoxic effect with IC_{50} value ≤ 6 µg/ml were subjected to apoptosis detection in the current study.

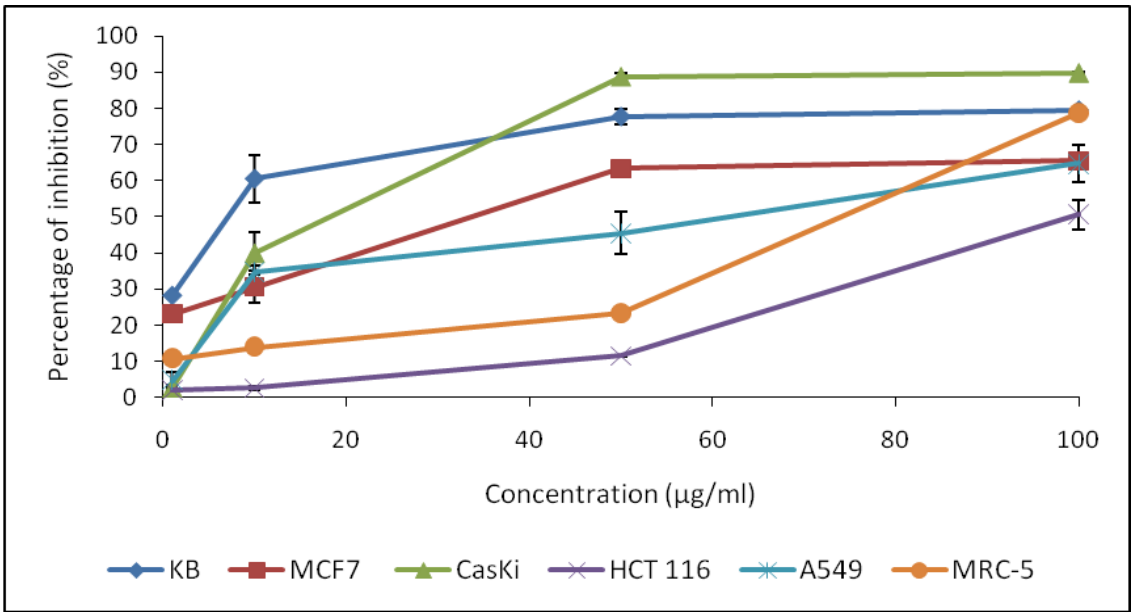


Figure 4.50: The *in vitro* growth inhibitions of phytol (3) against selected human cell lines determined by neutral red cytotoxicity assay. Each value is expressed as mean ± standard deviation of three measurements.

Table 4.22: Cytotoxic activity (IC_{50} values) of phytol (3) against selected human cell lines

Compound	Cytotoxicity (IC_{50}) in µg/ml (µM)					
	KB	MCF7	CasKi	HCT 116	A549	MRC-5
phytol (3)	7.1 (23.99)	34 (114.86)	18 (60.81)	100 (337.84)	31 (104.73)	74.3 (251.01)
Doxorubicin[a]	$1.3x10^{-2}$ (0.023)	$7.6x10^{-2}$ (0.139)	$6.0x10^{-3}$ (0.011)	$3.6x10^{-1}$ (0.663)	$2.2x10^{-1}$ (0.401)	$5.5x10^{-1}$ (1.01)

[a] Doxorubicin was used as the reference compound

4.9.4 Cytotoxic effect of ß-sitosterol (4)

ß-Sitosterol (**4**) was isolated from both *P. bleo* and *P. grandifolia*. The *in vitro* growth inhibitions of ß-sitosterol (**4**) against selected human cell lines are shown in Figure 4.51. The percentage inhibition was increased with increase in ß-sitosterol (**4**) concentrations. In the present study, β-sitosterol (**4**) did not display cytotoxic effects against the tested cell lines (Table 4.23). The results obtained here were in agreement with published data (Awad *et al*., 2000; Block *et al*., 2004; Bennani *et al*., 2007; Jackson *et al*., 2000; Moghadasian, 2000). There have been reports that plant sterols are able to stimulate estrogen dependent cancer cells *in vitro* (e.g. Ju *et al*., 2004). The MCF7 cell line used in this study was purchased from ATCC. It was reported that MCF7 cells from ATCC were unaffected by estrogen or antiestrogen (Osborne *et al*., 1987). Thus, the result showed that the sterols have weak inhibition on the growth of MCF7 cells.

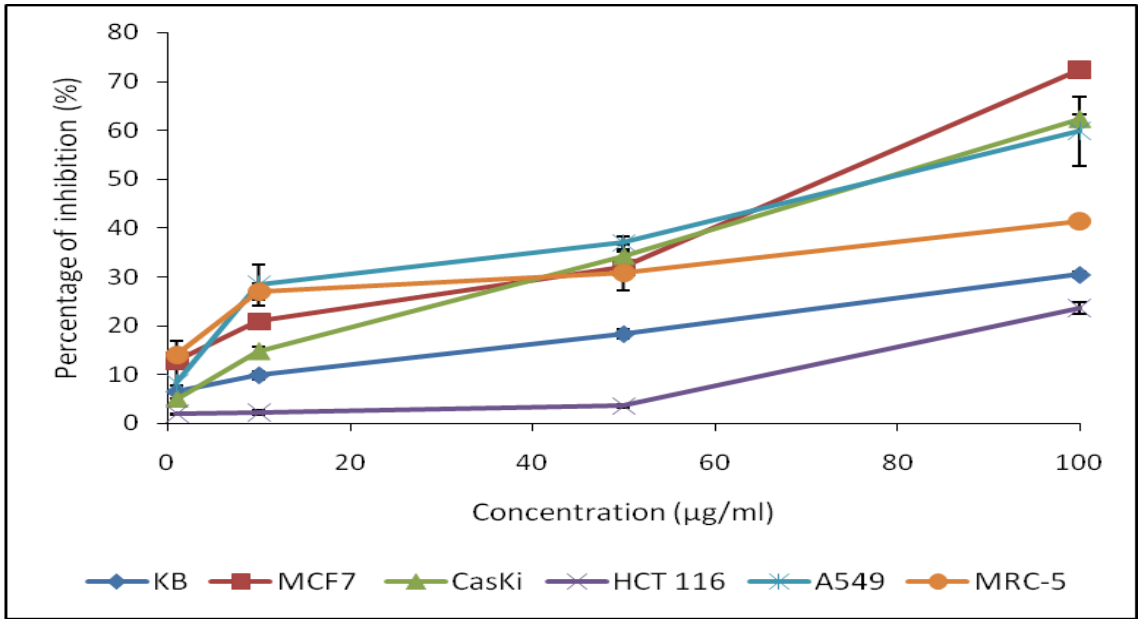


Figure 4.51: The *in vitro* growth inhibitions of ß-sitosterol (4) against selected human cell lines determined by neutral red cytotoxicity assay. Each value is expressed as mean ± standard deviation of three measurements.

Table 4.23: Cytotoxic activity (IC_{50} values) of ß-sitosterol (4) against selected human cell lines

Compound	Cytotoxicity (IC_{50}) in µg/ml (µM)					
	KB	MCF7	CasKi	HCT 116	A549	MRC-5
ß-Sitosterol (**4**)	> 100 (> 241.55)	72 (173.91)	62 (149.76)	> 100 (> 241.55)	78 (188.41)	> 100 (> 241.55)
Doxorubicin[a]	$1.3x10^{-2}$ (0.023)	$7.6x10^{-2}$ (0.139)	$6.0x10^{-3}$ (0.011)	$3.6x10^{-1}$ (0.663)	$2.2x10^{-1}$ (0.401)	$5.5x10^{-1}$ (1.01)

[a] Doxorubicin was used as the reference compound

4.9.5 Cytotoxic and apoptosis effects of dihydroactinidiolide (5)

(i) Cytotoxic activity of dihydroactinidiolide (5)

In this study, dihydroactinidiolide (**5**) demonstrated relatively strong cytotoxic effect against HCT 116 cells (IC_{50} 5.0 µg/ml; Table 4.24) in comparison with other human cell lines. The *in vitro* growth inhibitions of dihydroactinidiolide (**5**) against selected human cell lines are shown in Figure 4.52. Percentage of inhibition was increased with the increment in dihydroactinidiolide (**5**) concentrations.

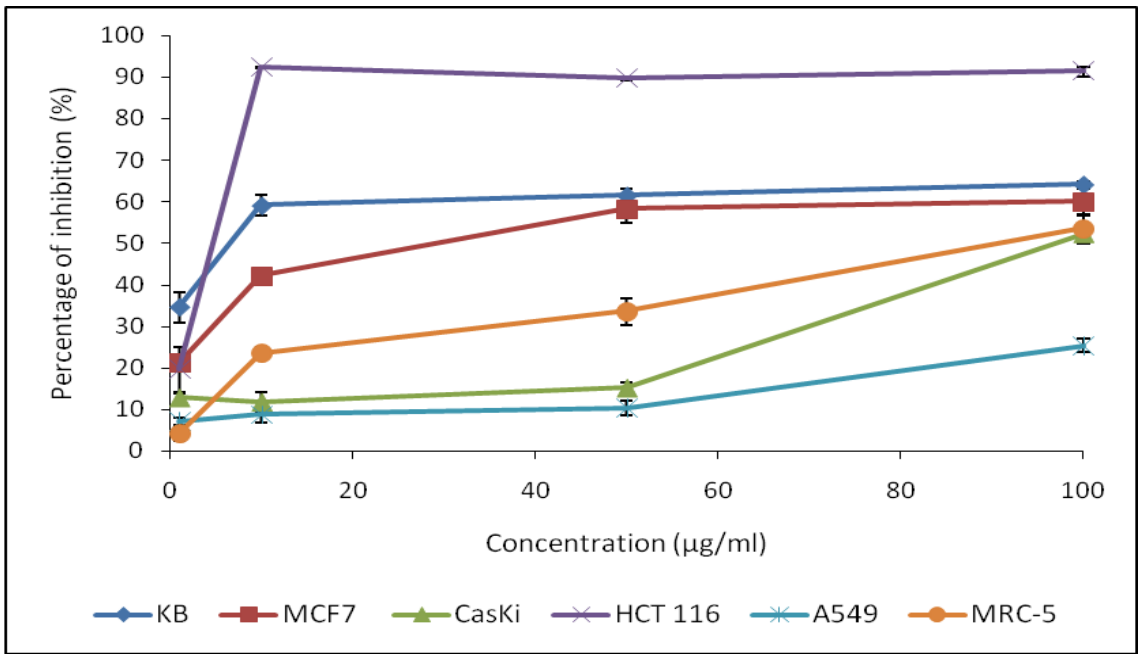


Figure 4.52: The *in vitro* growth inhibitions of dihydroactinidiolide (5) against selected human cell lines determined by neutral red cytotoxicity assay. Each value is expressed as mean ± standard deviation of three measurements.

Table 4.24: Cytotoxic activity (IC_{50} values) of dihydroactinidiolide (5) against selected human cell lines

Compound	Cytotoxicity (IC_{50}) in μg/ml (μM)					
	KB	MCF7	CasKi	HCT 116	A549	MRC-5
Dihydroactinidiolide (**5**)	6.7 (37.22)	30 (166.67)	40 (222.22)	**5** (27.78)	97 (538.89)	91.3 (507.22)
Doxorubicin[a]	$1.3x10^{-2}$ (0.023)	$7.6x10^{-2}$ (0.139)	$6.0x10^{-3}$ (0.011)	$3.6x10^{-1}$ (0.663)	$2.2x10^{-1}$ (0.401)	$5.5x10^{-1}$ (1.01)

[a] Doxorubicin was used as the reference compound

(ii) Induction of apoptosis by dihydroactinidiolide (5) on HCT 116 cells

According to Table 4.24, dihydroactinidiolide (**5**) demonstrated relatively strong cytotoxic effect against HCT 116 cells. In order to determine whether the cytotoxic effects of dihydroactinidiolide (**5**) was due to apoptosis, HCT 116 cells were treated with dihydroactinidiolide (**5**) for 24 h and modified TUNEL assay was performed. The nuclei of HCT 116 cells treated with dihydroactinidiolide (**5**) were stained dark brown, but very few in control cells.

(iii) Expression level of apoptosis-related genes in dihydroactinidiolide (5)-treated HCT 116 cells

Figure 4.53 shows the time dependency effects of p53, caspase-3 and c-myc mRNA levels in HCT 116 cells induced with dihydroactinidiolide (**5**). Figure 4.53 shows that the expression level of caspase-3 was increased drastically when the HCT 116 cells were treated with dihydroactinidiolide (**5**) at 15 min (4.7-fold increase as compared to control cells) and 30 min (20.3-fold increase as compared to control cells) and decreasing thereafter. The result here strongly indicates that dihydroactinidiolide (**5**)

killed the HCT 116 cells through apoptosis mechanism mainly *via* the activation of caspase-3 while the role of p53 and c-myc cannot be totally ruled out.

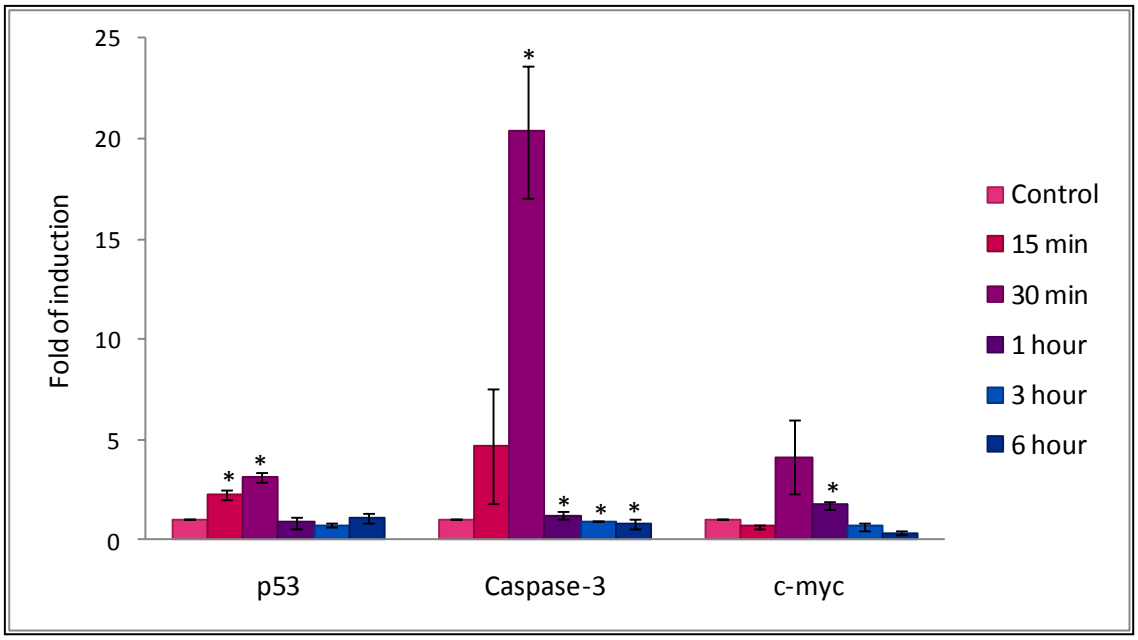


Figure 4.53: The mRNA expression of p53, caspase-3 and c-myc detected in HCT 116 cells treated with dihydroactinidiolide (5) for different durations. Results are expressed as means ± standard deviation. *: Significant difference from the control ($p < 0.05$).

4.9.6 Cytotoxic effect of sterol mixture (mixture A)

In the present study, the sterol mixture [campesterol (**6**), stigmasterol (**7**) and β-sitosterol (**4**)] did not display cytotoxic effects against the tested cell lines (Table 4.25). The *in vitro* growth inhibitions of sterol mixture against selected human cell lines are shown in Figure 4.54. The results obtained here were in agreement with published data (Awad *et al*., 2000; Block *et al*., 2004; Bennani *et al*., 2007; Jackson *et al*., 2000; Moghadasian, 2000).

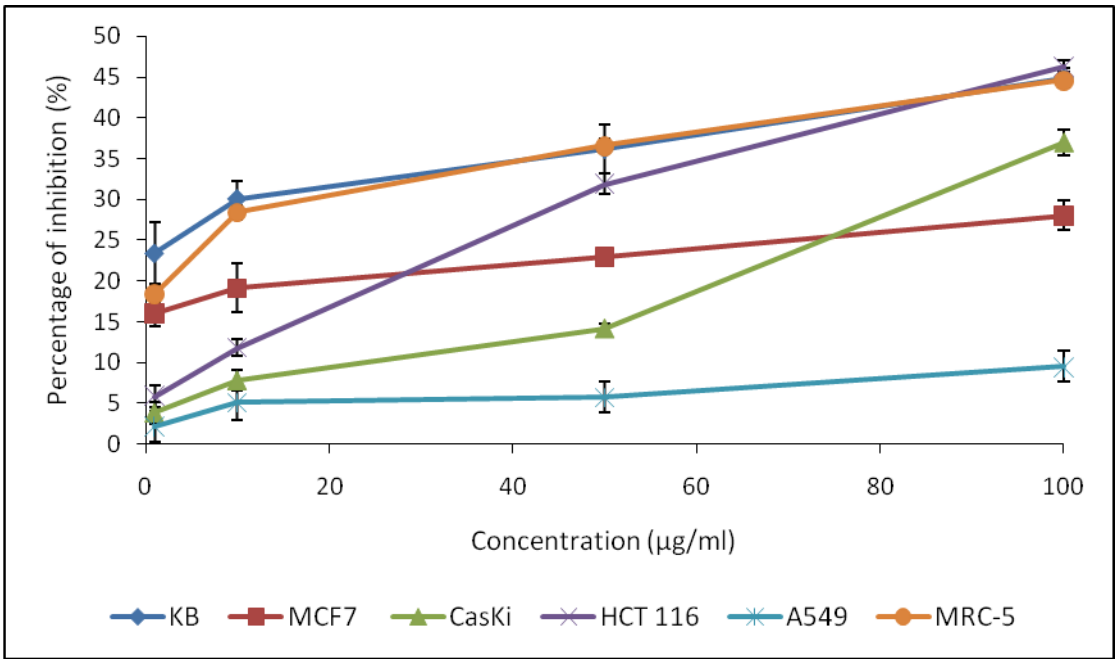


Figure 4.54: The *in vitro* growth inhibitions of sterol mixture (mixture A) against selected human cell lines determined by neutral red cytotoxicity assay. Each value is expressed as mean ± standard deviation of three measurements.

Table 4.25: Cytotoxic activity (IC_{50} values) of sterol mixture (mixture A) against selected human cell lines

Mixture	Cytotoxicity (IC_{50}) in µg/ml (µM)					
	KB	MCF7	CasKi	HCT 116	A549	MRC-5
Mixture of sterols (**Mixture A**)	> 100	> 100	> 100	> 100	> 100	> 100
Doxorubicin[a]	$1.3x10^{-2}$ (0.023)	$7.6x10^{-2}$ (0.139)	$6.0x10^{-3}$ (0.011)	$3.6x10^{-1}$ (0.663)	$2.2x10^{-1}$ (0.401)	$5.5x10^{-1}$ (1.01)

[a] Doxorubicin was used as the reference compound

4.9.7 Cytotoxic and apoptosis effects of phytone (8)

(i) Cytotoxic activity of phytone (8)

In the present study, phytone (**8**) was isolated from the ethyl acetate extract of *P. grandifolia*. As shown in Table 4.26, phytone (**8**) demonstrated pronounced activity against CasKi and HCT 116 cells (IC_{50} 6 and 5 µg/ml, respectively). The *in vitro* growth inhibitions of phytone (**8**) against selected human cell lines are shown in Figure 4.55. Percentage of inhibition was increased with increase in phytone (**8**) concentrations.

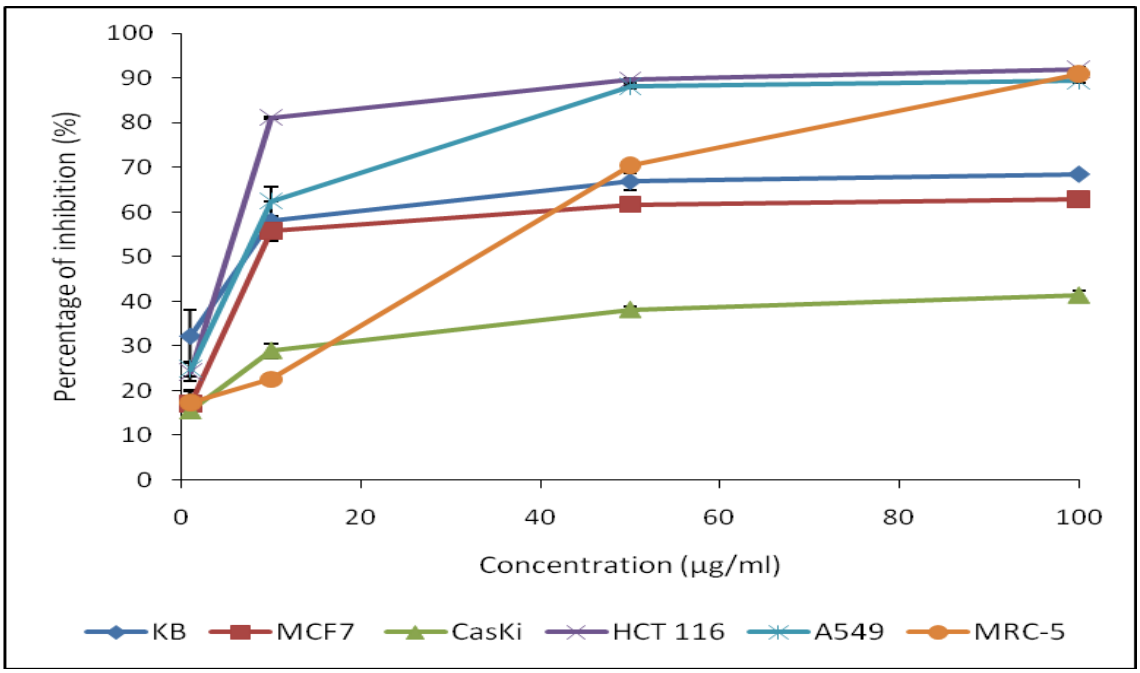


Figure 4.55: The *in vitro* growth inhibitions of phytone (8) against selected human cell lines determined by neutral red cytotoxicity assay. Each value is expressed as mean ± standard deviation of three measurements.

Table 4.26: Cytotoxic activity (IC_{50} values) of phytone (8) against selected human cell lines

Compound	Cytotoxicity (IC_{50}) in µg/ml (µM)					
	KB	MCF7	CasKi	HCT 116	A549	MRC-5
Phytone **(8)**	7.2 (26.87)	8.7 (32.46)	6 (22.39)	5 (18.66)	7 (26.12)	31.3 (116.79)
Doxorubicin[a]	$1.3x10^{-2}$ (0.023)	$7.6x10^{-2}$ (0.139)	$6.0x10^{-3}$ (0.011)	$3.6x10^{-1}$ (0.663)	$2.2x10^{-1}$ (0.401)	$5.5x10^{-1}$ (1.01)

[a] Doxorubicin was used as the reference compound

(ii) Induction of apoptosis by phytone (8) on selected cells

In order to determine whether the cytotoxic effects of phytone (**8**) was apoptotic in nature, CasKi and HCT 116 cells were treated with phytone (**8**) for 24 h and modified TUNEL assay (DeadEnd™ Colorimetric Apoptosis Detection System, Promega) was performed. The nuclei of CasKi and HCT 116 cells treated with phytone (**8**) were stained dark brown while none of the cell nucleus was stained dark in the negative controls.

(iii) Expression level of apoptosis-related genes in phytone (8)-treated cells

CasKi cell line

Figure 4.56 shows the time dependency effects of p53, caspase-3 and c-myc mRNA levels in CasKi cells induced with phytone (**8**). A significantly strong up-regulation ($p < 0.05$) in p53 expression was observed. As shown in Figure 4.56, the mRNA level of p53 was significantly increased ($p < 0.05$) at 15 min (314.5-fold

increase as compared to control cells), 30 min (543.4-fold increase as compared to control cells) and 1 h (763.9-fold increase as compared to control cells). However, the expression level was significantly dropped ($p < 0.05$) to 14.7-fold at 3 h but significantly increased ($p < 0.05$) again to 115.7-fold at 6 h. On the other hand, significantly up-regulation ($p < 0.05$) was also observed in caspase-3 and c-myc expression (Figure 4.57). However, the expression level of caspase-3 and c-myc was not as high as p53 (Figure 4.57). Thus, the results strongly indicate that phytone (**8**) killed the CasKi cells through apoptosis mechanism *via* the activation of p53 while the role of caspase-3 cannot be totally ruled out.

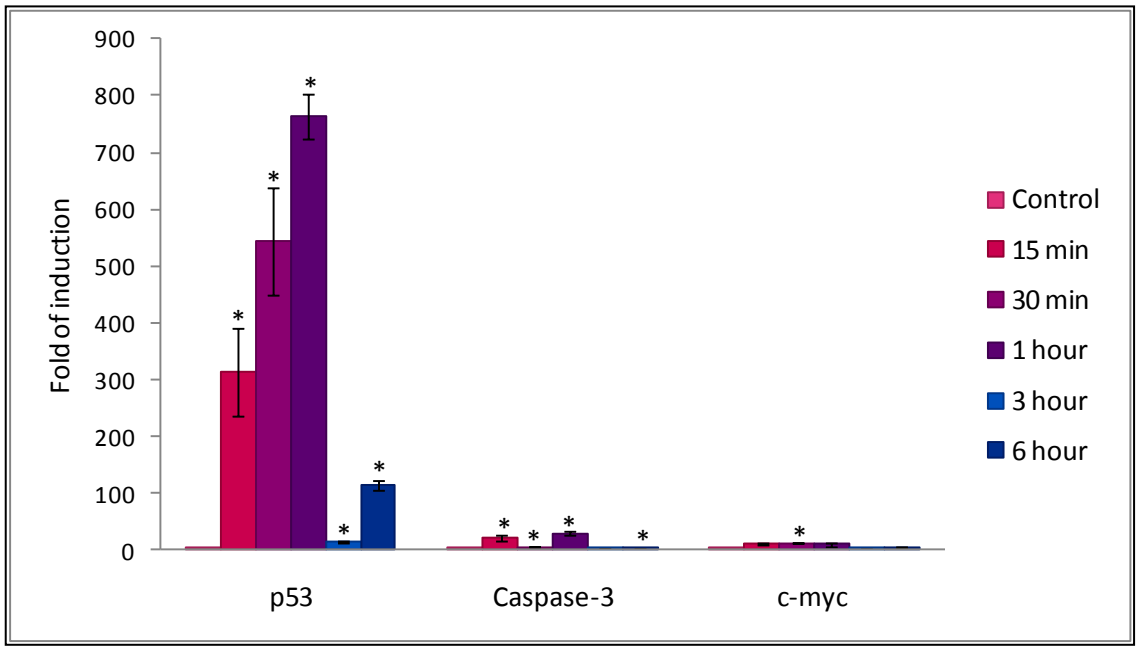


Figure 4.56: The mRNA expression of p53, caspase-3 and c-myc detected in CasKi cells treated with phytone (8) for different durations. Results are expressed as means ± standard deviation. *: Significant difference from the control ($p < 0.05$).

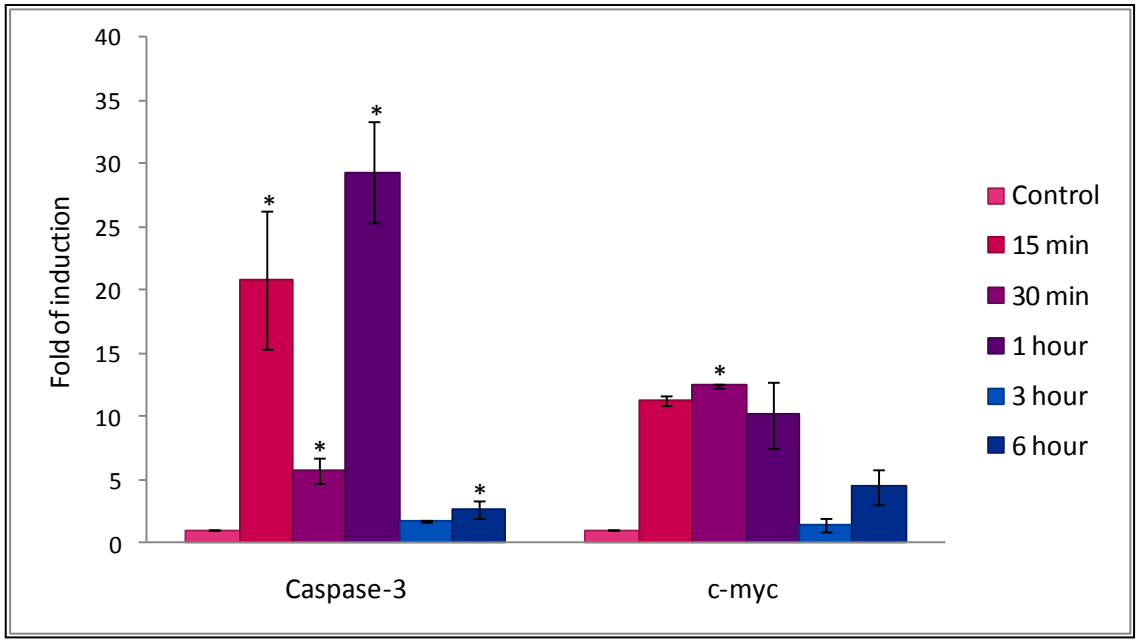


Figure 4.57: The mRNA expression of caspase-3 and c-myc detected in CasKi cells treated with phytone (8)for different durations. Results are expressed as means ± standard deviation. *: Significant difference from the control ($p < 0.05$).

HCT 116 cell line

Figure 4.58 shows the time dependency effects of p53, caspase-3 and c-myc mRNA levels in HCT 116 cells induced with phytone (**8**). The expression of p53, caspase-3 and c-myc were highest at 30 min, with 2.8-, 2.7- and 1.6-fold increase as compare to the control cells, respectively. Interestingly, the gene expression pattern of p53, caspase-3 and c-myc mRNA in treated-HCT 116 cells was similar, which the mRNA expression levels were down-regulated at 15 min, up-regulated at 30 min and down-regulated again at 3 h. The results here thus suggest that the apoptotic effects of phytone (**8**) in the HCT 116 cells were associated with p53, caspase-3 and c-myc activation.

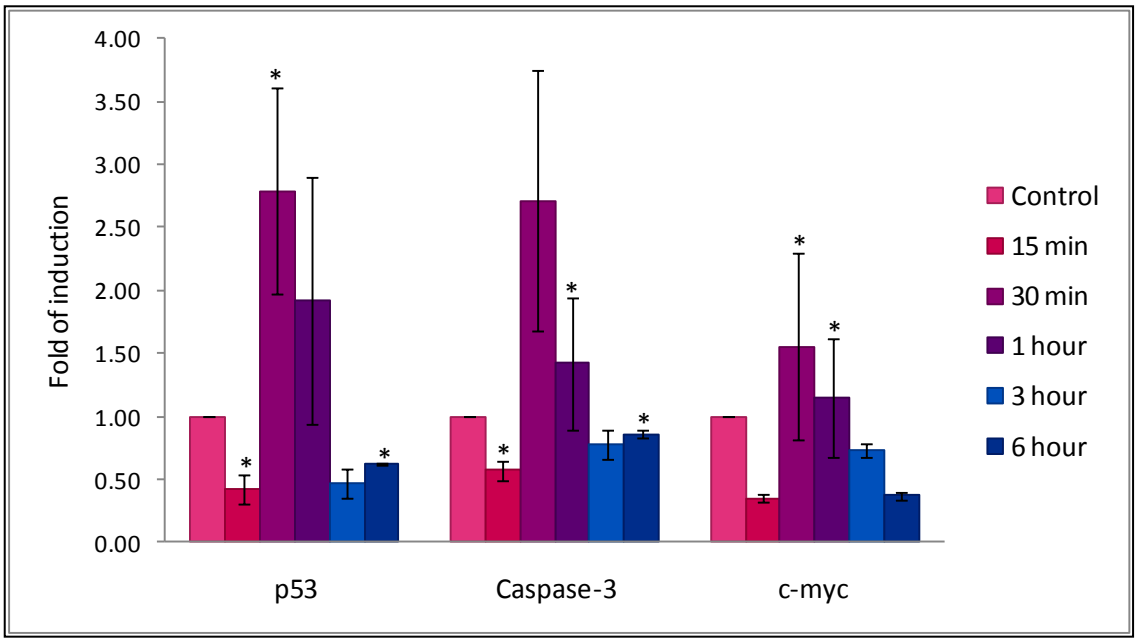


Figure 4.58: The mRNA expression of p53, caspase-3 and c-myc detected in HCT 116 cells treated with phytone (8) for different durations. Results are expressed as means ± standard deviation. *: Significant difference from the control ($p < 0.05$).

4.9.8 Cytotoxic and apoptosis effects of mixture B

(i) Cytotoxic activity of mixture B

As shown in Table 4.20, 2,4-di-tert-butylphenol (**1**) displayed very remarkable cytotoxic activity against KB cells with IC_{50} value of 0.81 µg/ml and strong cytotoxicity against MCF7 (IC_{50} 5.75 µg/ml), A549 (IC_{50} 6 µg/ml) and CasKi cells (IC_{50} 4.5 µg/ml). Surprisingly, as shown in Table 4.27, the mixture B which consisted of 2,4-ditert-butylphenol (**1**), methyl palmitate (**9**), methyl oleate (**10**) and methyl stearate (**11**) showed promising cytotoxic effects against CasKi (IC_{50} 6 µg/ml) and A549 (IC_{50} 5 µg/ml). However, they also displayed cytotoxic activity against normal MRC-5 cells with IC_{50} 6.9 µg/ml. 2, 4-Di-tert-butylphenol (**1**) is the major component (80.82 %) in the mixture.

The cytotoxicity of the mixture against the MRC-5 normal cells is not contributed by this compound as in its pure form, it only exert a mild effect on the

MRC-5 normal cell line (IC_{50} 20 µg/ml). The cytotoxicity of this mixture may have been contributed by other components in the mixture. The data reported here thus suggest that methyl esters might exert cytotoxic effect against normal MRC5 cells but not on KB, MCF7 and HCT 116 cells.

This finding on cytotoxicity of methyl esters is supported by Takeara *et. al* (2008) who reported that methyl palmitate exerted cytotoxic effect on T-cell leukemia cell line (Molt-4) with IC_{50} value of 2.28 µg/ml while methyl stearate was cytotoxic to acute promyeloblastic leukemia cell line (HL-60) and Molt-4 cell line with IC_{50} values of 3.08 and 4.65 µg/ml, respectively.

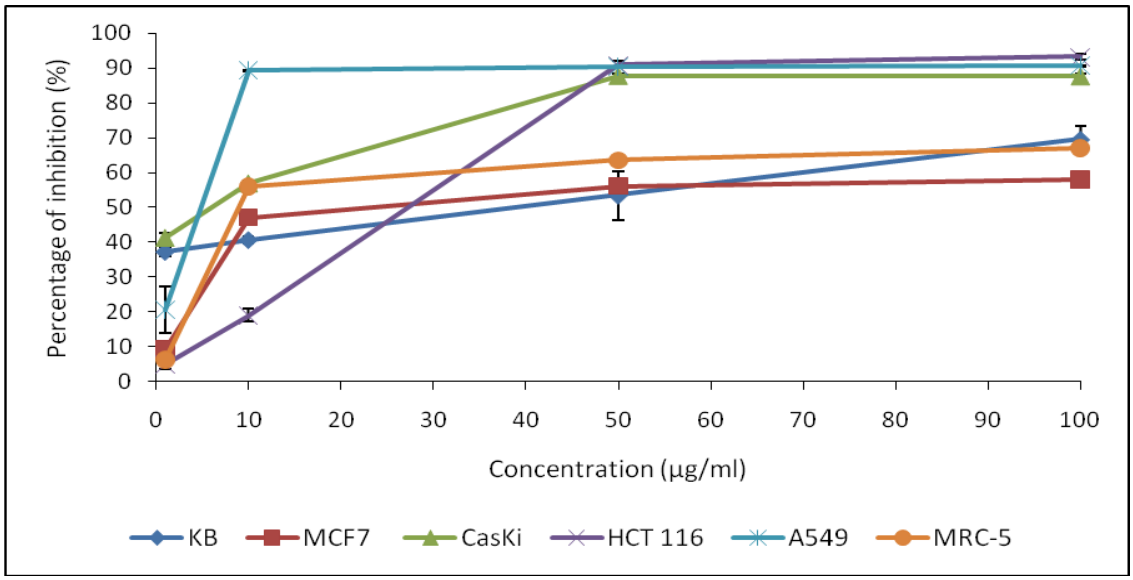


Figure 4.59: The *in vitro* growth inhibitions of mixture B against selected human cell lines determined by neutral red cytotoxicity assay. Each value is expressed as mean ± standard deviation of three measurements.

Table 4.27: Cytotoxic activity (IC_{50} values) of mixture B against selected human cell lines

Compound	Cytotoxicity (IC_{50}) in µg/ml (µM)					
	KB	MCF7	CasKi	HCT 116	A549	MRC-5
Mixture B	40	23.5	**6**	28	**5**	6.9
Doxorubicin[a]	$1.3x10^{-2}$ (0.023)	$7.6x10^{-2}$ (0.139)	$6.0x10^{-3}$ (0.011)	$3.6x10^{-1}$ (0.663)	$2.2x10^{-1}$ (0.401)	$5.5x10^{-1}$ (1.01)

[a] Doxorubicin was used as the reference compound

(ii) Induction of apoptosis by mixture B on selected cells

According to Table 4.27, mixture B demonstrated pronounced activity against CasKi and A549 cells (IC_{50} 6 and 5 µg/ml respectively). In order to determine whether the cytotoxic effects of mixture B was due to apoptosis, CasKi and A549 cells were treated with mixture B for 24 h and modified TUNEL assay (DeadEnd™ Colorimetric Apoptosis Detection System, Promega) was performed to detect the presence of apoptosis. The nuclei of CasKi and A549 cells treated with mixture B were stained dark brown. However, none of the cell nucleus was stained dark in the negative controls.

(iii) Expression level of apoptosis-related genes in mixture B-treated cells

CasKi cell line

Figure 4.60 shows the time dependency effects of p53, caspase-3 and c-myc mRNA levels in CasKi cells induced with **mixture B**. The expression of p53, caspase-3 and c-myc were highest at 6 h (32.5-fold increase as compare to the control cells), 3 h (7.5-fold increase as compare to the control cells) and 6 h (11.1-fold increase as compare to the control cells), respectively. The expression level of p53 was down-

regulated at 15 min and 30 min, but drastically up-regulating thereafter. The results here indicate that mixture B killed the CasKi cells through apoptosis mechanism mainly *via* the activation of p53 although the role of caspase-3 and c-myc cannot be ruled out.

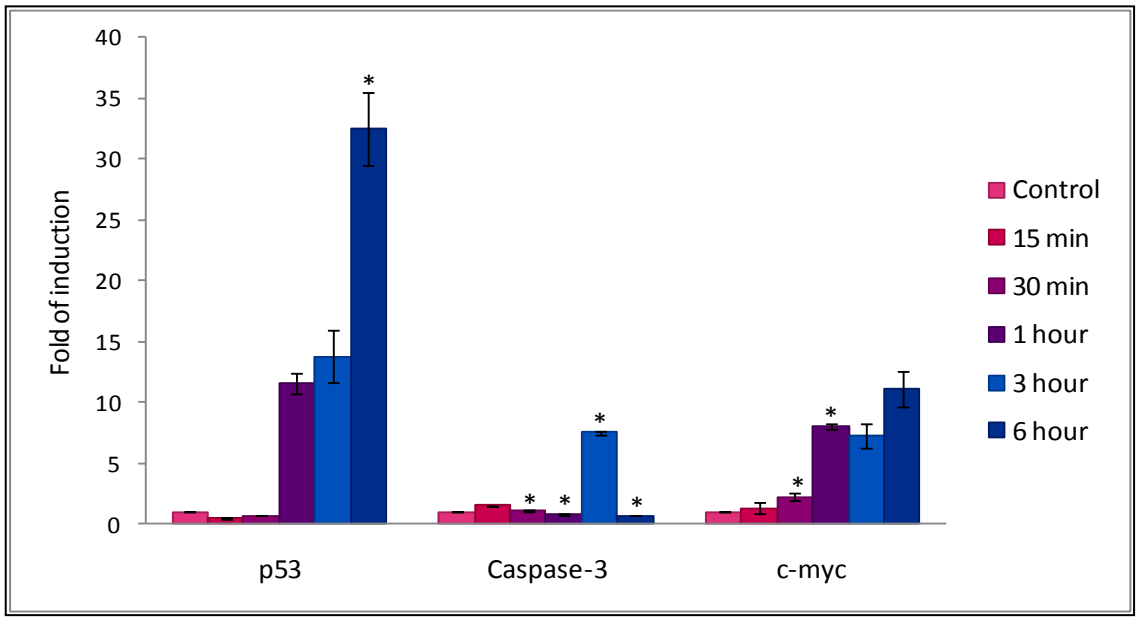


Figure 4.60: The mRNA expression of p53, caspase-3 and c-myc detected in CasKi cells cells treated with mixture B for different durations. Results are expressed as means ± standard deviation. *: Significant difference from the control ($p < 0.05$).

A549 cell line

Figure 4.61 shows the time dependency effects of p53, caspase-3 and c-myc mRNA levels in A549 cells induced with mixture B. The up-regulation in p53, caspase-3 and c-myc expressions were observed. The expression of p53, caspase-3 and c-myc were highest at 3 h (2.0-fold increase as compare to the control cells), 1 h (3.1-fold increase as compare to the control cells) and 3 h (1.7-fold increase as compare to the control cells), respectively. The result here strongly indicates that mixture B killed the A549 cells through apoptosis mechanism mainly *via* the activation of p53, caspase-3 and c-myc.

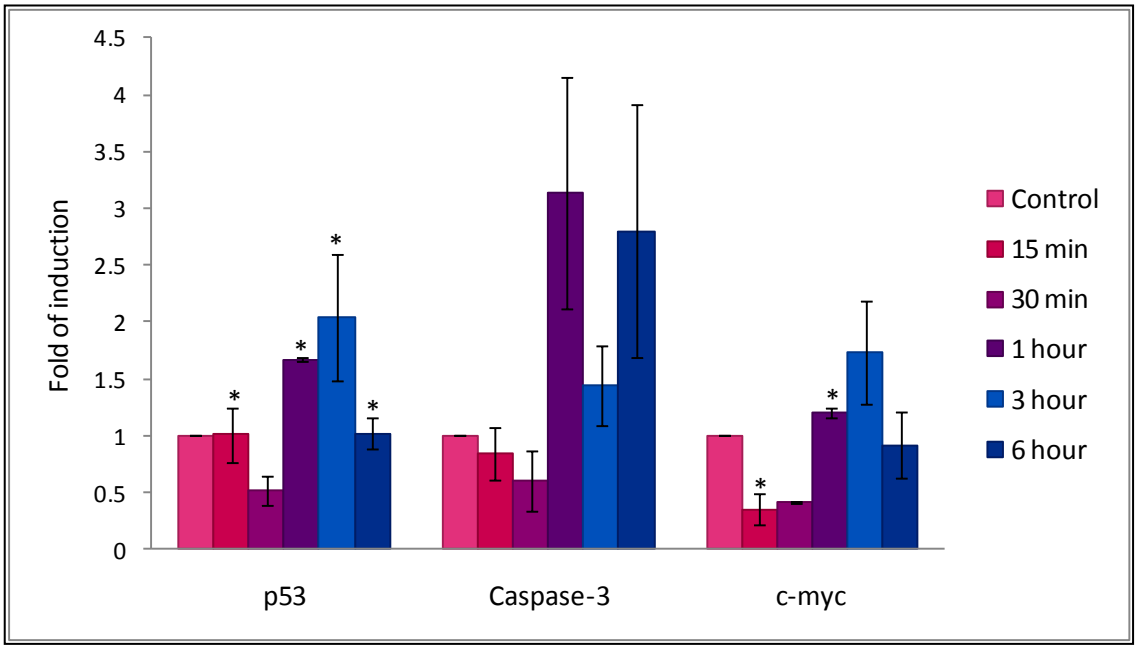


Figure 4.61: The mRNA expression of p53, caspase-3 and c-myc detected in A549 cells treated with mixture B for different durations. Results are expressed as means ± standard deviation. *: Significant difference from the control ($p < 0.05$).

4.9.9 Comparison of cytotoxic and apoptosis effects of chemical constituents isolated from the bioactive extracts of *P. bleo* and *P. grandifolia*

The results of cytotoxicity screening of the chemical constituents are summarized in Table 4.28. As shown in Table 4.28 and Table 4.29, the chemical constituents present in a particular bioactive extract have different activity. The observed activity in the extract might be due to synergism between chemical constituents present in the extract. The synergism among these chemical constituents which contribute to the cytotoxic activity is not only dependent on the concentration of the constituents, but also on the structure and interaction(s) between the constituents (Houghton and Raman, 1998). This can explain the differences in the cytotoxic effect between crude extracts and isolated compounds against the same cell lines. For example, the cytotoxic effect of the *P. bleo* methanol extract (Table 4.29) on the KB cell lines showed an IC_{50} of 6.5 µg/ml and such impressive activity was supported by some of the isolated compounds [dihydroactinidiolide (**5**), 2,4-di-tert-butylphenol (**1**),

α-tocopherol (**2**) and phytol (**3**)]. In contrast, the cytotoxic effect of the crude methanol extract of *P. bleo* on the MCF7 cell line (Table 4.29) gave IC_{50} of 39.0 µg/ml (mild) whilst two isolated compounds 2,4-di-tert-butylphenol (**1)** and α-tocopherol (**2**), showed good inhibitory activities with IC_{50} values of 5.75 and 7.5 µg/mL, respectively.

In summary, the cytotoxic activity observed for ethyl acetate extract of *P. bleo* (section 4.4) was ascribable to the presence of the cytotoxic active compounds 2,4-di-tert- butylphenol (**1**), α-tocopherol (**2**), phytol (**3**) and dihydroactinidiolide (**5**). On the other hand, the cytotoxic activity observed for ethyl acetate extract of *P. grandifolia* (section 4.4) was ascribable to the active compounds 2,4-di-tert- butylphenol (**1**), α-tocopherol (**2**), phytone (**7**) and mixture **B**.

Based on the cytotoxicity result (Table 4.28), although the cytotoxic activities of these chemical constituents were not as effective as doxorubicin, in comparison to the latter they have low toxicity against normal MRC5 cell line (Table 4.28). The use of the isolated chemical constituents as single anticancer agents would not merit consideration. However, their use in combination with cytotoxic therapeutic drugs might reduce the adverse effects of some of these drugs. Support for this suggestion is provided by Amir *et al.* (2007), who reported that in addition to having potent antitumor properties as single agents, natural products have also demonstrated potential synergy with established cytotoxic therapeutic drugs in pre-clinical studies. At this stage, it is not possible to justify the use of isolated compounds in comparison to doxorubicin in the treatment of cancer. A more comprehensive investigation is required.

In addition, the selected human cancer cells treated with isolated chemical constituents clearly demonstrated DNA fragmentation when detected using modified TUNEL assay (DeadEnd^{TM} Colorimetric Apoptosis Detection System, Promega),

indicating apoptotic cell death as the major mechanism involved. Apoptosis detection is further supported by gene expression studies whereby the mRNA expression levels of three apoptotic-related genes, c-myc, p53 and caspase-3 in cells treated with chemical constituents were investigated. It was found out that the apoptosis elicited by the isolated chemical constituents on selected cancer cells was mediated by c-myc, p53 and caspase-3 in different expression levels. These findings were in agreement with many studies that demonstrated the role of c-myc, p53 and caspase-3 in inducing apoptosis.

Table 4.28: Cytotoxic activity (IC_{50} values) of chemical constituents isolated from the bioactive ethyl acetate extracts of *P. bleo* and *P. grandifolia*

Compound	Cytotoxicity (IC_{50}) in µg/m (µM)					
	KB	MCF7	CasKi	HCT 116	A549	MRC-5
2,4-Di-tert- butylphenol (**1**)	**0.81** (3.93)	**5.75** (27.91)	**4.5** (21.84)	29 (140.78)	**6** (29.13)	20 (97.09)
α-Tocopherol (**2**)	8 (18.60)	7.5 (17.44)	**6** (13.95)	31 (72.09)	**6** (13.95)	30.5 (70.93)
Phytol (**3**)	7.1 (23.99)	34 (114.86)	18 (60.81)	100 (337.84)	31 (104.73)	74.3 (251.01)
β -Sitosterol (**4**)	>100 (>241.55)	72 (173.91)	62 (149.76)	> 100 (> 241.55)	78 (188.41)	> 100 (> 241.55)
Dihydroactinidiolide (**5**)	6.7 (37.22)	30 (166.67)	40 (222.22)	**5** (27.78)	97 (538.89)	91.3 (507.22)
Mixture A	> 100	> 100	> 100	> 100	> 100	> 100
Phytone (**7**)	7.2 (26.87)	8.7 (32.46)	**6** (22.39)	**5** (18.66)	7 (26.12)	31.3 (116.79)
Mixture B	40	23.5	**6**	28	**5**	6.9
Doxorubicin[a]	$1.3x10^{-2}$ (0.023)	$7.6x10^{-2}$ (0.139)	$6.0x10^{-3}$ (0.011)	$3.6x10^{-1}$ (0.663)	$2.2x10^{-1}$ (0.401)	$5.5x10^{-1}$ (1.01)

[a] Doxorubicin was used as the reference compound

Table 4.29: Cytotoxic activity (IC_{50} values) of bioactive extracts and chemical constituents isolated from the bioactive ethyl acetate extract of *P. bleo*

Extract / Compound	Cytotoxicity (IC_{50}) in µg/m (µM)					
	KB	MCF7	CasKi	HCT 116	A549	MRC-5
Methanol extract	**6.5**	40.5	41.0	39.0	56	61.0
Ethyl acetate extract	**4.5**	58.0	22.0	28.0	41	> 100.0
2,4-Di-tert- butylphenol (**1**)	**0.81** (3.93)	**5.75** (27.91)	**4.5** (21.84)	29 (140.78)	**6** (29.13)	20 (97.09)
α-Tocopherol (**2**)	8 (18.60)	7.5 (17.44)	**6** (13.95)	31 (72.09)	**6** (13.95)	30.5 (70.93)
Phytol (**3**)	7.1 (23.99)	34 (114.86)	18 (60.81)	100 (337.84)	31 (104.73)	74.3 (251.01)
β -Sitosterol (**4**)	>100 (>241.55)	72 (173.91)	62 (149.76)	> 100 (> 241.55)	78 (188.41)	> 100 (> 241.55)
Dihydroactinidiolide (**5**)	6.7 (37.22)	30 (166.67)	40 (222.22)	**5** (27.78)	97 (538.89)	91.3 (507.22)
Mixture A	> 100	> 100	> 100	> 100	> 100	> 100
Doxorubicin[a]	$1.3x10^{-2}$ (0.023)	$7.6x10^{-2}$ (0.139)	$6.0x10^{-3}$ (0.011)	$3.6x10^{-1}$ (0.663)	$2.2x10^{-1}$ (0.401)	$5.5x10^{-1}$ (1.01)

[a] Doxorubicin was used as the reference compound

4.10 Screening for alkaloids of *P. bleo* and *P. grandifolia*

Doetsch *et al.* (1980) reported the isolation of four alkaloids, namely 3,4-dimethoxy-β-phenethylamine, mescaline, 3-methoxytyramine and tyramine from *P. bleo* and three alkaloids, *p*-methoxy-β-hydroxy-β-phenethylamine, 3-methoxytyramine and tyramine from *P. grandifolia* (Figure 2.12). *P. bleo* and *P. grandifolia* used in the study of Doetsch *et al.* (1980) was collected from Fairchild Tropical Gardens, Miami,

Fla., USA and botanical gardens, University of Michigan, Ann Arbor, Mich, USA respectively. On the other hand, the *Pereskia* species in this study was collected from Selangor, Malaysia. The locally grown *P. bleo* and *P. grandifolia* were also screened for alkaloids in the present study to confirm the presence of alkaloids.

In the preliminary alkaloidal test, a few drops of Wagner's reagent were added to an aliquot containing plant extract, a (++) for definite turbidity in *P. bleo* and (+) for slight turbidity in *P. grandifolia* were observed. However, the result recorded was preliminary evidence that alkaloids are present, a confirmatory test was then conducted. When a few drops of Wagner's reagent were added to the filtrate, (+) for slight turbidity was observed in *P. bleo* and *P. grandifolia*. A (+) result recorded was indicative of the presence of primary, secondary or tertiary alkaloids.

Test for quaternary and/ or amine oxide bases was pursued. A few drops of Wagner's reagent were added to a solution of extract giving a (++) for definite turbidity in *P. bleo* and (+) for slight turbidity in *P. grandifolia*. The (++) and (+) result observed in the test may be taken as an indication of the presence of quaternary and/ or amine oxide bases.

In summary, the screening indicated the presence of alkaloids in locally grown *Pereskia spp.* but the concentration was very low as (+) result was recorded in the confirmatory test of alkaloids. Thus, extraction and isolation of alkaloids was not pursued in the present study. It is highly probably the environment and location in which plants of the same species were found has a profound effect on the concentration levels of a particular secondary metabolites (in this case the alkaloids).

CHAPTER 5

CONCLUSION

P. bleo and *P. grandifolia* have been traditionally used for treatment of cancer-related diseases by locals. The findings of the current study thus provide scientific validation on the use of the leaves of *P. bleo* and *P. grandifolia*. In view of the increasing popular consumption of medicinal plants as alternative therapy, it is therefore necessary to conduct serious research to support the therapeutic claims and also to ensure that the plants are indeed safe for human consumption. The present research findings have clearly met the objectives of the study.

The experimental approach in the present study is based on bioassay-guided fractionation. In this endeavor, the crude methanol and fractionated extracts of *P. bleo* and *P. grandifolia* were firstly prepared for biological assessment, such as antioxidant, antimicrobial and neutral red cytotoxicity assays to identify the bioactive extracts of both *Pereskia spp*.

For *P. bleo*, the ethyl acetate extract showed the highest reducing capacity measured by Folin-Ciocalteau method and the highest antioxidant activity in ß-carotene bleaching assay significantly ($p < 0.05$). The hexane extract of *P. bleo* showed significantly ($p < 0.05$) the highest antioxidant activity when determined by the scavenging effect on DPPH radicals and reducing power assay.

For *P. grandifolia*, the reducing capacity, DPPH scavenging and the ß-carotene bleaching activities of the ethyl acetate extract was significantly the highest among the extracts ($p < 0.05$). On the other hand, the hexane extract of *P. grandifolia* possessed significantly ($p < 0.05$) highest reducing power capacity among the extracts.

Based on the antioxidant assays, it is thus suggested that the antioxidant activities of hexane extracts in both *Pereskia spp*. were not solely contributed by phenolic compounds but other components in the extracts. The hexane extracts of both *Pereskia spp*. were found to contain mainly methyl esters. It is highly probable that the lone pairs of electron on the carbonyl oxygen can be easily donated to the ferric ions in the reducing power assay. Methyl palmitate, methyl linoleate, methyl α-linolenate and phytol were identified from the hexane extract of *P. bleo* by GCMS analysis whilst methyl palmitate, methyl linoleate, methyl α-linolenate and methyl stearate were identified from the hexane extract of *P. grandifolia*

Next, the extracts of both *Pereskia spp.* did not demonstrate promising antimicrobial activities against the tested microorganisms, except the ethyl acetate extracts of both *Pereskia spp.* which showed some mild activities against the tested bacteria. The results suggested that there might be some active antimicrobial components in the extracts but present in low quantity and were not present in sufficient concentrations to be effective. The results of *P. bleo* here were thus in agreement with Ruegg *et al.* (2006).

Subsequently, the extracts of *P. bleo* and *P. grandifolia* were investigated for their cytotoxic effects against selected human cell lines, namely the Human Nasopharyngeal Epidermoid Carcinoma Cell Line (KB), Human Cervical Carcinoma Cell Line (CasKi), Human Colon Carcinoma Cell Line (HCT 116), Hormone-Dependent Breast Carcinoma Cell Line (MCF7) and non-cancer Human Fibroblast Cell Line (MRC-5) by using an *in vitro* neutral red cytotoxicity assay.

The ethyl acetate extracts of both *Pereskia spp.* in general gave higher inhibition and stimulation values against various cancerous cell lines compared to other

extracts. The ethyl acetate extract of *P. bleo* was selectively toxic against KB cells. Both of the ethyl acetate extracts displayed the distinctively cytotoxicity effect on KB cell line, which reached IC_{50} at relatively low concentration. The ethyl acetate extract of *P. bleo* has a higher activity (IC_{50} value against KB cells of 4.5 μg/ml) in comparison to the ethyl acetate extract of *P. grandifolia* (IC_{50} 16.0 μg/ml). Ethyl acetate extract of *P. grandifolia*, on the other hand displayed a good inhibition against MCF7 cells with IC_{50} of 20.0 μg/ml. The active ingredients in ethyl acetate extract may lead to valuable compounds that may have the ability to kill KB cancer cells but exert no damage to normal cells (IC_{50} > 100.0 μg/ml against normal cells, MRC-5).

The hexane extract of *P. grandifolia* also demonstrated a remarkably high inhibition towards KB cells with IC_{50} value of 5 μg/ml, in comparison to that of *P. bleo*. The water extracts of both *Pereskia spp.* were found to have no effect on the cancer cell lines (IC_{50} > 100.0 μg/ml in all cases). All the extracts of both *Pereskia spp.* demonstrated weaker cytotoxicity profile against the CaSki and A549 cells compared to other cells and no activity against normal cells (IC_{50} > 100.0 μg/ml).

In addition, cell deaths of the selected cancer cells elicited by the cytotoxic active extracts of both *Pereskia spp*. were found to be apoptotic in nature based on a clear indication of DNA fragmentation, which is a hallmark of apoptosis. The LUX RT-qPCR analysis showed the mRNA expression levels of c-myc were markedly increased in the cancer cells treated with the cytotoxic active extracts while the p53 and caspase-3 expression were only slightly increased as compared to c-myc in all cases, except for the *P. grandifolia* ethyl acetate extract in KB cells. The apoptotic effects of the *P. grandifolia* ethyl acetate extract in KB cells were associated with increases in all three c-myc, p53 and caspase-3 activation. The results thus suggested that the cytotoxic

active extracts of *P. bleo* and *P. grandifolia* contained bioactive compounds capable of inducing apoptosis in selected cancer cell lines.

From the results of the biological screenings of both *Pereskia spp*., it is observed that the hexane and ethyl acetate extracts generally have stronger biological activities than the crude methanol extracts. This was probably due to the increase in concentration of active chemical constituents in the fractionated extract as a consequence of fractionation. Further chemical investigations were thus directed to the ethyl acetate extracts of both *Pereskia spp*. Fractionation into hexane and then ethyl acetate extraction was avoided as the yield of ethyl acetate extracts obtained was very low.

Chemical investigations of the bioactive ethyl acetate extracts led to the isolation and identification of some chemical constituents. For *P. bleo*, 2,4-di-tert-butylphenol (**1**), α-tocopherol (**2**), phytol (**3**), ß-sitosterol (**4**), dihydroactinidiolide (**5**) and a mixture of sterols [mixture A; campesterol (**6**), stigmasterol (**7**) and β-sitosterol (**4**)] were obtained. 2,4-Di-tert-butylphenol (**1**), α-tocopherol (**2**), phytol (**3**) and plant sterols [particularly ß-sitosterol (**4**)] may have contributed to the reducing capacity and antioxidant activity of the ethyl acetate extract of *P. bleo*. Whilst, the mild antimicrobial activity of ethyl acetate extract of *P. bleo* might be due to the presence of 2,4-di-tert-butylphenol (**1**), ß-sitosterol (**4**) and the mixture of sterols (mixture **A**).

2,4-Di-tert-butylphenol (**1**), α-tocopherol (**2**), ß-sitosterol (**4**) and the mixture [mixture B; methyl palmitate (**9**), methyl oleate (**10**), methyl stearate (**11**) and 2,4-di-tert-butylphenol (**1**)] were isolated from *P. grandifolia*. 2,4-Di-tert-butylphenol (**1**), α-tocopherol (**2**) and ß-sitosterol (**4**) may have contributed to the antioxidant activity of the ethyl acetate extract of *P. grandifolia*. The mild antimicrobial activity of the ethyl

acetate extract of *P. grandifolia* maybe due to 2,4-di-tert-butylphenol (**1**) and ß-sitosterol (**4**).

It is interesting to note that 2,4-ditert-butylphenol (**1**), α-tocopherol (**2**) and ß-sitosterol (**4**) were isolated from both *Pereskia spp*. The cytotoxic activities of the isolated constituents were evaluated against five human carcinoma cell lines, namely KB, CasKi, HCT 116, MCF7 and A549 cell lines; and non-cancer human fibroblast cell line (MRC-5) using the neutral red cytotoxicity assay. The chemical constituents demonstrated cytotoxic activity were further detected with DNA fragmentation and further subjected to the gene expression study.

According to the cytotoxicity results, the cytotoxic activity observed for the ethyl acetate extract of *P. bleo* was ascribable to the presence of the cytotoxic active compounds 2,4-di-tert-butylphenol (**1**), α-tocopherol (**2**), phytol (**3**) and dihydroactinidiolide (**5**). On the other hand, the cytotoxic activity observed for ethyl acetate extract of *P. grandifolia* was ascribable to the active compounds 2,4-di-tert-butylphenol (**1**), α-tocopherol (**2**), phytone (**7**) and mixture **B**.

Although the cytotoxic activities of these chemical constituents were not as effective as doxorubicin, in comparison to the latter they have low toxicity against normal MRC5 cell line. Although the isolated chemical constituents cannot be used as single anticancer agents, but their use in combination with established cytotoxic therapeutic drugs might reduce the adverse effects of some of these drugs.

Furthermore, the selected human cancer cells treated with cytotoxic active chemical constituents clearly demonstrated DNA fragmentation when detected using modified TUNEL assay, indicating apoptotic cell death as the major mechanism involved. This was further supported by investigation on the mRNA expression levels

of three apoptotic-related genes, c-myc, p53 and caspase-3 in cells treated with chemical constituents, in order to determine the mechanism of sample-induced apoptosis. It was found out that the apoptosis elicited by the isolated chemical constituents on selected cancer cells was mediated by c-myc, p53 and caspase-3 giving different expression levels. These findings were consistent with many studies that demonstrated the role of c-myc, p53 and caspase-3 in inducing apoptosis.

The crude methanol extracts of both *Pereskia spp.* were also evaluated for the acute oral toxicity to determine the safety parameters of both *Pereskia spp.* The result obtained was in agreement to that of *in vitro* experiments, further confirming the results that crude extracts of *P. bleo* and *P. grandifolia* did not show cytotoxicity against normal cells. Thus, the crude extracts of both *Pereskia spp.* were considered to be safe in acute oral toxicities in experimental mice.

The alkaloid screening was also performed to confirm the presence of alkaloids as reported by Doetsch *et al.* (1980). The alkaloid screening of the locally grown *P. bleo* and *P. grandifolia* indicated the presence of alkaloids, but the concentration was very low. Thus, extraction and isolation of alkaloids was not pursued in the present study. The difference of alkaloid concentration in the studies might be due to the plant material was collected from different places.

Lastly, a protocol for the evaluation of mRNA expression levels of apoptotic genes by real-time reverse transcriptase–polymerase chain reaction (RT-qPCR) using LUX (Light Upon eXtension) primers has been established in the present study. The LUX RT-qPCR assay significantly simplifies and accelerates the process of producing reproducible quantification of mRNAs. The mRNAs were reverse transcribed, amplified, detected and quantitated in real time. However, this technique has not been

commonly employed in natural product field and there are no reports so far on LUX RT-qPCR methods for gene expression study on natural products. Thus, the protocol developed can be recommended for gene expression study in natural products.

In conclusion, the findings of *P. bleo* and *P. grandifolia* in the present study support the common belief that ethnopharmacological selection of *P. bleo* and *P. grandifolia* is a useful criterion in drug discovery. As suggestions, further studies on the mutagenic and toxicity effect over a longer period of time involving detection of effects on vital organ functions should be carried out to ensure that the plants are safe for human consumption.

REFERENCES

Abas, F. (2005). *Phytochemical and biological activity studies of Cosmos caudatus and Curcuma mangga and the online characterization of bioactive fractions from Melicope ptelefolia*. Unpublished doctoral dissertation, Universiti Putra Malaysia, Malaysia.

Abravaya, K., Huff, J., Marshall, R., Merchant, B., Mullen, C., Schneider, G. and Robinson, J. (2003). Molecular beacons as diagnostic tools: technology and application. *Clinical Chemistry and Laboratory Medicine, 41* (4), 468-474.

Adams, R.P. (2001). *Identification of essential oil components by gas chromatography/quadrupole mass spectroscopy*. USA: Allured Publishing Corporation.

Adrain, C. and Martin, S.J. (2001). The mitochondrial apoptosome: A killer unleashed by the chromosome seas. *Trends in Biochemical Sciences, 26*, 390-397.

Aerts, J.L., Gonzales, M.I. and Topalian, S.L. (2004). Selection of appropriate control genes to assess expression of tumour antigens using real-time RT-PCR. *Biotechniques, 36* (1), 84-91.

Agarwala, S. and Kalil, R.E. (1998). Axotomy-induced neuronal death and reactive astrogliosis in the lateral geniculate nucleus following a lesion of the visual cortex in the rat. *The Journal of Comparative Neurology, 392*, 252-263.

Ahmad, I and Beg, A.Z. (2001). Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. *Journal of Ethnopharmacology, 74*, 113-123.

Ahmad, I., Mehmood, Z. and Mohammad, F. (1998). Screening of some Indian medicinal plants for their antimicrobial properties. *Journal of Ethnopharmacology, 62*, 183-193.

Ahmad, S. (1995). *Oxidative stress and antioxidant defenses in biology*. New York: Chapman & Hall.

Ahn, H.Y., Hadizadeh, K.R., Seul, C., Yun, Y.P., Vetter, H. and Sachinidis, A. (1999). Epigallocathechin-3 gallate selectively inhibits the PDGF-BB-induced intracellular signaling transduction pathway in vascular smooth muscle cells and inhibitis transformation of sis-transfected NIH 3T3 fibroblasts and human glioblastoma cells (A172). *Molecular Biology of the Cell, 10*, 1093-1094.

Aitichou, M.J.S. and Ibrahim, M. (2005). Two-colour multiplex assay for the identification of orthopox viruses with real-time LUX-PCR. *Molecular and Celullar Probes, 19* (5), 323-328.

Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W. and Yuan, J. (1996). Human ICE/CED-3 protease nomenclature. *Cell, 87*, 171.

Alonso, R., Fernansez-Aranguiz, A., Colom, K., Herreras, A. and Cisterna, R. (2000). Profile of bacterial isolates and antimicrobial susceptibility: multicentre study using a one-day cut-off. *Revista Espanola de Quimioterapia*, *13*, 384-393.

Al-Sherbini, E-S. A.M., El Noury, A.H., and El Rouby, M.N. (2009). Vitamin E (α-tocopherol) enhances the PDT action of hematoporphyrin derivatives on cervical cancer cells. *Medical Laser Application*, *24*, 65-73.

Alves, C., C. Pio and A. Duarte. (2001). Composition of extractable organic matter of air particles from rural and urban Portugese areas. *Atmospheric Environment*, *35*, 5485-5496.

Amarowicz, R., Pegg, R.B., Moghaddam, P.R., Barl, B. and Weil, J.A. (2004). Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian Prairies. *Food Chemistry*, *84* (4), 551-562.

Ames, B.N. (1983). Dietary carcinogens and anticarcinogens. *Science*, *221*, 1256-1264.

Ames, B.N. and Shigenaga, M.K. (1992). Oxidants are a major contributor to aging. *Annals of the New York Academy of Sciences*, *663*, 85-96.

Amir, D., Sunil, K., Gautama, S. and Bharat, B. A. (2007). Back to basics: How natural products can provide the basis for new therapeutics. *Expert Opinion on Investigational Drugs*, *16*, 1753-1773.

Anon. (1969). *Wealth of India* (Vol. VII, pp. 309). India: Council of Scientific & Industrial Research.

Ansari, B., Coates, P.J., Greenstein, B.D. and Hall, P.A. (1995). *In situ* end-labeling detects DNA strand breaks in apoptosis and other physiological and pathological states. *Journal of Pathology*, *170*, 1-8.

Aoki, K., Ishiwata, S., Sakagami, H., Kusama, K. and Katayama, T. (2001). Modification of apoptosis-inducing activity of gallic acid by saliva. *Anticancer Research*, *21*, 1879-1883.

Arends, M.J., Morris, R.G. and Wyllie, A.H. (1990). Apoptosis: The role of the endonuclease. *American Journal of Pathology*, *136*, 593-608.

Aruoma, O.I. (1994). Nutrition and health aspects of free radicals and antioxidants. *Food and Chemical Toxicology*, *32* (7), 671-683.

Askew, O.S., Ashmun, R.A., Simmons, B.C. and Cleveland, J.L. (1991). Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. *Oncogene*, *6*, 1915-1922.

Awad, A.B., Downie, A.C. and Fink, C.S. (2000). Inhibition of growth and stimulation of apoptosis by β-sitosterol treatment of MDA-MB-231 human breast cancer cells in culture. *International Journal of Molecular Medicine*, *5*, 541-545.

Ayyoubi, S.A. and Muhtasib, H.G. (2007). Differential apoptosis by gallotannin in human colon cancer cells with distinct p53 status. *Molecular Carcinogenesis, 46*, 176-186.

Barthlott, W. and D.R., Hunt. (1993). Cactaceae. In K. Kubitzki, J.G. Rohwer and V. Bittrich (Eds.), *The families and genera of vascular plants* (pp. 161-197). Berlin: Springer Verlag.

Becker, E.M., Nissen, L.R. and Skibsted, L.H. (2004). Antioxidant evaluation protocols: food quality or health effects. *European Food Research and Technology*, *219* (6), 561-571.

Bektic, J., Guggenberger, R., Eder, I.E., Pelzer, A.E., Berger, A.P., Bartsch, G. (2005). Molecular effects of the of the isoflavonoid genistein in prostate cancer. *Clinical Prostate Cancer*, *4*, 124-129.

Bennani, H., Drissi, A., Giton, F., Kheuang, L., Fiet, J. and Adlouni, A. (2007). Antiproliferative effect of polyphenols and sterols of virgin argan oil on human prostate cancer cell lines. *Cancer Detection and Prevention*, *31*, 64-69.

Benson, L. (1982)**.** *The cacti of the United States and Canada*. California: Stanford University Press.

Benzie, F.F.I. and Strain, J.J. (1999). Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and ascorbic acid concentration. *Methods in Enzymology*, *299*, 15-27.

Benzie, I.F. (2000). Evolution of antioxidant defence mechanisms. *European Journal of Nutrition*, *39* (2), 53-61.

Bera, D., Lahiri, D. and Nag, A. (2006). Studies on a natural antioxidant for stabilization of edible oil and comparison with synthetic antioxidants. *Journal of Food Engineering*, *74*, 542-545.

Berahou, A., Auhmani, A., Fdil, N., Benharref, A., Jana, M. and Gadhi, C.A. (2007). Antibacterial activity of *Quercus ilex* bark's extracts. *Journal of Ethnopharmacology*, *112*, 426-429.

Berger, A., Jones, P.J.H. and Abumweis, S.S. (2004). Plant sterols: Factors affecting their efficacy and safety as functional food ingredients. *Lipids in Health and Disease, 3*, 5-24.

Berliner, J.A. and Heinecke, J.W. (1996). The role of oxidized lipoproteins in atherogenesis. *Free Radical Biology and Medicine, 20*, 707.

Bermudez, Y., Ahmadi, S., Lowell, N.E. and Kruk, P.A. (2007). Vitamin E suppresses telomerase activity in ovarian cancer cells. *Cancer Detection and Prevention*, *31*, 119-128.

Block, S., Baccelli, C., Tinant, B., Meervelt, L.V., Rozenberg, R., Jiwan, J.L., Habib, L., Gabriel, P.G., De, M.C. and Joelle, Q.L. (2004). Diterpenes from the leaves of *Croton zambesicus*. *Phytochemistry*, *65*, 1165-1171.

Blois, M.S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature*, *181*, 1199-1200.

Boik, J. (2001). *Natural compounds in cancer therapy*. Minnesota, USA: Oregon Medical Press.

Borenfreund, E. and Puerner, J.A. (1984). A simple quantitative procedure using monolayer culture for toxicity assays. *Journal of Tissue Culture Methods*, *9*, 7-9.

Borenfreund, E. and Puerner, J.A. (1985). Toxicity determined in vitro by morphological alterations and neutral red absorption. *Toxicology Letters*, *24*, 119-124.

Borenfreund, E. and Puerner, J.A. (1986). Cytotoxicity of metals, metal-metal and metal-chelator combinations assayed *in vitro*. *Toxicology*, *39*, 131-134.

Borse, B.B., Rao, L.J.M., Nagalakshmi, S. and Krishnamurthy, N. (2002). Fingerprint of black teas from India: identification of the regio-specific characteristics. *Food Chemistry, 79*, 419-424.

Boscoboinik, D., Szewczyk, A., Hensey, C. and Azzi, A. (1991). Inhibition of cell proliferation by alpha-tocopherol.role of protein kinase C. *Journal of Biological Chemistry, 266* (10), 6188-6194.

Botterweck, A.A.M., Verhagen, H., Goldbohm, R.A., Kleinjans, J. and Brandt, P.A. (2000). Intake of butylated hydroxyanisole and butylated hydroxytoluene and stomach cancer risk: results from analyses in the Netherlands cohort study. *Food and Chemical Toxicology*, *38*, 599-605.

Bouic, P.J. (2001). The role of phytosterols and phytosterolins in immune modulation: a review of the past 10 years. *Current Opinion in Clinical Nutrition and Metabolic Care*, *4*, 471-475.

Bowen, I.D., Bowen, S.M. and Jones, A.H. (1998). *Mitosis and apoptosis: Matters of life and death*. London: Chapman and Hall.

Brian, L., Herbert, A.A., Bloom, F.R., Gleeson, M. and Kusser, W. (2003). Quarantitation of gene expression in neural precursors by reverse-transcription polymerase chain reaction using self-quenched, fluorogenic primers. *Analytical Biochemistry*, *319* (1), 177.

Brook, I. (1994). Otitis media: Microbiology and management. *Journal of Otolaryngology*, *23*, 269-275.

Brook, I and Gober, A.E. (1998). Microbiological characteristics of persistent otitis media. *Archives of Otolaryngology-Head and Neck Surgery*, *124*, 1350-1352.

Bustin, S.A. (2000). Absolute quantification of mRNA using real-time reverse transcriptase polymerase chain reaction assay. *Journal of Molecular Endocrinology, 25*, 169-193.

Bustin, S.A., Benes, V., Nolan, T. and Pfaffl, M.W. (2005). Quantitative real-time RT-PCR- a perspective. *Journal of Molecular Endocrinology*, *34*, 597-601.

Campbell, S.E., Stone, W.L., Lee, S., Whaley, S., Yang, H., Qiu, M., Goforth, P., Sherman, D., McHaffie, D. and Krishnan, K. (2006). Comparative effects of RRR-alpha- and RRR-gamma-tocopherol on proliferation and apoptosis in human colon cancer cell lines. *BMC Cancer*, *6*, 13.

Cao, G., Alessio, H.M. and Cutler, R.G. (1993). Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biology and Medicine*, *14*, 303-311.

Cao, G., Booth, S.L., Sadowski, J.A. and Prior, R.L. (1998). Increases in human plasma antioxidant capacity after consumption of controlled diets high in fruits and vegetables. *The American Journal of Clinical Nutrition*, *68*, 1081-1087.

Cappelletty, D. (1998). Microbiology of bacterial respiratory infections. *Pediatric Infectious Disease Journal*, *17*, S55-S61.

Cardellina, J.H., Fuller, R.W., Gamble, W.R., Westergaard, C., Boswell, J., Munro, M.H.G., Currens, M. and Boyd, M.R. (1999). Evolving strategies for the selection, dereplication and prioritization of antitumour and HIV-inhibitory natural products extracts. In L. Bohlin and J.G. Bruhn (Eds.), *Bioassay methods in natural product research and development* (pp. 25-36). Dordrecht: Kluwer Academic Publishes.

Cardone. M.H., Roy, N., Stennicke, H.R., Salvesen, G.S., Franke, T.F., Stanbridge, E., Frisch, S. and Reed, J.C. (1988). Regulation of cell death protease caspase-9 by phosphorylation. *Science*, *282*, 1318-1321.

Carter, S.K.A. (1975). A review. *Journal of the National Cancer Institute*, *55*, 1256-1274.

Chang, L.W., Yen, W.J., Huang, S.C. and Duh, P.D. (2002). Antioxidant activity of sesame coat. *Food Chemistry*, *78*, 347–354.

Chapuis, J.C., Sordat, B. and Hostettmann, K. (1988). Screening for cytotoxic activity of plants used in traditional medicine. *Journal of Ethnopharmacology*, *23*, 273-284.

Charriaut-Marlangue, C. and Ben Ari, Y. (1995). A cautionary note on the use of the TUNEL stain to determine apoptosis. *Neororeport*, *7*, 61.

Chen, R., Huang, W., Lin, Z., Zhou, Z., Yu, H. and Zhu, D. (2004). Development of a novel real-time RT-PCR assay with LUX primer for the detection of swine transmissible gastroenteritis virus. *Journal of Virological Methods*, *122*, 57-61.

Chen, W.Y. (2002). *Kesan kinetin dan BAP terhadap penggandaan pucuk Jarum Tujuh Bilah (Pereskia bleo) secara in vitro*. Unpublished BSc.'s thesis, Universiti Putra Malaysia, Malaysia.

Chen, Y.J., Shiao, M.S., Hsu, M.L., Tsai, T.H. and Wang, S.Y. (2001). Effect of caffeic acid phenethyl ester, an antioxidant from propolis, on inducing apoptosis in human leukemic HL-60 cells. *Journal of Agricultural and Food Chemistry*, *49*, 5615-5619.

Cheung, L.M., Cheung, P.C.K. and Ooi, V.E.C. (2003). Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chemistry*, *81*, 249-255.

Choi, Y. and Lee, J. (2009). Antioxidant and antiproliferative properties of a tocotrienol-rich fraction from grape seeds. *Food chemistry, 114*, 1386-1390.

Clemedson, C. and Ekwall, B. (1999). Overview of the final MEIC results: The *in vitro-in vitro* evaluation. *Toxicology in Vitro, 13*, 657-663.

Cook, J.A. and Mitchell, J.B. (1989). Viability measurements in mammalian cell systems. *Analytical Biochemistry, 179*, 1-7.

Cordell, G.A., Beecher, C.C.W. and Pezzuto, J.M. (1991). Can ethnopharmacology contribute to the development of new cancer drugs? *Journal of Ethnopharmacology, 32*, 117-133.

Cosulich, S.C., Horiuchi, H., Zerial, M., Clarke, P.R. and Woodman, P.G. (1997). Cleavage of rabaptin-5 blocks endosome fusion during apoptosis. *EMBO Journal, 16*, 6182-6191.

Cragg, G.M., Boyd, M.R., Cardellina, I.I., Newman, D.J., Snader, K.M. and McCloud, T.G. (1994). Ethnobotany and drug discovery: the experience of the US National Cancer Institute. In Ciba Foundation 185, *Ethnobotany and search for new drugs*. Chichester: John Wiley and Sons.

Cunningham, M.W. (2000). Pathogenesis of group A streptococcal infections. *Clinical Microbiology Reviews, 13*, 470-511.

Dandona, P., Thusu, K., Cook, S., Snyder, B. and Nicotera, T. (1996). Oxidative DNA damage diabetes mellitus. *Lancet, 347*, 444-445.

Darmstadt, G.L. and Lane, A.T. (1994). Impetigo: An overview. *Pediatric Dermatology, 11*, 293-303.

Davis, J. (1994). Inactivation of antibiotics and the dissemination of resistance genes. *Science, 264*, 375-382.

Davison, F.D., Groves, M. and Scaravilli, F. (1995). The effects of formalin fixation on the detection of apoptosis in human brain by *in situ* end-labelling of DNA. *Histochemical Journal, 27*, 983.

DeBiasi, R.L. and Tyler, K.L. (2004). Molecular methods for diagnosis of viral encephalitis. *Clinical Microbiology Reviews, 17* (4), 903-925.

Deciga-Campos, M., Rivero-Cruz, I., Arriaga-Alba, M., Castaneda-Corral, G., Angeles-Lopez, G.E., Navarrete, A., Mata, R. (2007). Acute toxicity and mutagenic activity of Mexican plants used in traditional medicine. *Journal of Ethnopharmacology, 110*, 334-342.

Dedoussis, G.V.Z., Kaliora, A. and Andrikopoulos, N.K. (2005). Effect of phenols on natural killer (NK) cell-mediated death in the K562 human leukemic cell line. *Cell Biology International, 29*, 884-889.

Deorukhkar, A., Krishnan, S., Sethi, G and Aggarwal, B.B. (2007). Back to basics: how natural products can provide the basis for new therapeutics. *Expert Opinion on Investigational Drugs*, *16* (11), 1753-1773.

DeRenzis, F.A. and Schechtman, A. (1973). Staining by neutral red and trypan blue in sequence for assaying vital and nonvital cultured cells. *Stain Technology, 48*, 135-136.

Destoumieux, D., Bulet, P., Strub, J.-M., van Dorsselaer, A. and Bachere, E. (1999). Recombinant expression and range of activity of penaeidins, antimicrobial peptides from penaeid shrimp. *European Journal of Biochemistry*, *266*, 335-346.

Dheda, K., Huggett, J.F., Bustin, S.A., Johnson, M.A., Rook, G. and Zumla, A. (2004). Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *Biotechniques*, *37* (1), 112-119.

Doetsch, P.W., Cassady, J.M. and McLaughlin, J.L. (1980). Cactus alkaloids: XL. Identification of mescaline and other β-phenethylamines in *Pereskia*, *Pereskiopsis* and *Islaya* by use of fluorescamine conjugates. *Journal of Chromatography A*, *189*, 79-85.

Dorman, H.J.D., Kosar, M., Kahlos, K., Holm, Y. and Hiltunen, R. (2003). Antioxidant properties and composition of aqueous extracts from *Mentha* species, hybrids, varieties, and cultivars. *Journal of Agricultural and Food Chemistry, 51*, 4563-4569.

Drisko, J.A., Chapman, J. and Hunter, V.J. (2003). The use of antioxidant therapies during chemotherapy. *Gynecologic Oncology, 88*, 434-439.

Duan, X.-J., Zhang, W.-W., Li, X.-M. and Wang, B.-G. (2006). Evaluation of antioxidant property of extract and fractions obtained from a red alga, *Polysiphonia Urceolata*. *Food Chemistry*, *95* (1), 37-43.

Duh, P.D., Tu, Y.Y. and Yen, G.C. (1999). Antioxidant activity of water extract of Harug Jyur (*Chrysanthemum morifolium* Ramat). *Lebensmittel-Wissenchaft und Technologie*, *32***,** 269–277.

Duke, J.A. and Ayensu, E.S. (1985). *Medicinal plants of China*. MI: Research PublAlgonac.

Earnshaw, W.C., Martins, L.M. and Kaufmann, S.H. (1999). Mammalian caspases: structure, activation, substrates and functions during apoptosis. *Annual Review of Biochemistry*, *68*, 383-424.

Eckert, K.A. and Kunkel, T.A. (1991). DNA polymerase fidelity and the polymerase chain reaction. *PCR Methods and Applications*, *1*, 17-24.

Eidman, K.F. and MacDougall, B.S. (2006). Synthesis of loliolide, actinidiolide, dihydroactinidiolide, and aeginetolide *via* cerium enolate chemistry. *Journal of Organic Chemistry, 71*, 9513-9516.

Ellis, R.E., Yuan, J.Y. and Horvitz, H.R. (1991). Mechanisms and functions of cell death. *Annual Review of Cell and Development Biology*, *7*, 663-698.

Erel, O. (2004). A novel automated method to measure total antioxidant response against potent free radical reactions. *Clinical Biochemistry, 37* (2), 112-119.

Er, H.M., Chen, E. and Radhakrishnan. (2007). Anti-proliferative and mutagenic activities of aqueous and methanol extracts of leaves from *Pereskia bleo* (Kunth) DC leaves. *Journal of Ethnopharmacology, 113*, 448-456.

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A. and Nagata, S. (1998). A caspase-activated DNase that degrades DNA during apoptosis and its inhibitor ICAD. *Nature, 391*, 43-50.

Esterbauer, H., Dieber-Rhotheneder, M., Waeg, G., Striegl, G. and Jurgens, G. (1990). Biochemical, structural and functional properties of oxidized low-density lipoproptein. *Chemical Research in Toxicology, 3*, 77-92.

Evan, G.I., Wyllie, A.H., Gilbert, C.S., Littlewood, T.D., Land, H., Brooks, M., Waters, C.M., Penn, L.Z. and Hancock, D.C. (1992). Induction of apoptosis in fibroblasts by c-myc protein. *Cell*, *69*, 119-128.

Evans, W.C. (1996). *Trease and Evan's Pharmacognosy* (14th ed.). London: Bailliere Tindall.

Ezoubeiri, A., Gadhi, C.A., Fdil, N., Benharref, A., Jana, M. and Vanhaelen, M. (2005). Isolation and antimicrobial activity of two phenolic compounds from *Pulicaria odora* L. *Journal of Ethnopharmacology, 99*, 287-292.

Figueiredo, R., A.I. Rodrigues and M.C. Costa. (2007). Volatile composition of red clover (*Trifolium pratencse* L.) forages in Portugal: The influence of riping stage and ensilage. *Food Chemistry*, *104*, 1445-1453.

Floyd, R.A. (1990). The role of 8-hydroxyguanosine in carcinogenesis. *Carcinogenesis, 11*, 1447-1450.

Fotakis, G. and Timbrell, J.A. (2006). *In vitro* cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology Letters*, *160*, 171-177.

Foti, M.C., Daquino, C. and Geraci, C. (2004). Electron-transfer reaction of cinnamic acids and their methyl esters with the DPPH radical in alcoholic solutions. *Journal of Organic Chemistry, 69*, 2309-2314.

Fraga, C.G., Shigenega, M.K., Park, J., Deagan, P. and Ames, B.N. (1990). Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine. *The proceedings of the National Academy of Science USA, 87*, 4533-4537.

Frankel, E. and Meyer, A.S. (2000). The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *Journal of the Science of Food and Agriculture*, *80*, 1925-1941.

Freeman, W.M., Walker, S.J. and Vrana, K.E.(1999). Quantitative RT-PCR: Pitfalls and potential. *Biotechniques*, *26* (1), 112-122, 124-125.

Fujisawa, S., Atsumi, T., Kadoma, Y., Ishihara, M., Ito, S. and Yokoe, I. (2004). Kinetic radical scavenging activity and cytotoxicity of 2-methoxy- and 2-t-butyl-substituted phenols and their dimmers. *Anticancer Research*, 24 (5A), 3019-3026.

Furiga, A., Lonvaud-Funel, A. and Badet, C. (2009). *In vitro* study of antioxidant capacity and antibacterial activity on oral anaerobes of a grape seed extract. *Food Chemistry*, *113*, 1037-1040.

Galal, M., Bashir, A.K., Salih, A.M. and Adam, S.E.I. (1991). Activity of water extracts of *Albiziaanthelmintica* and *A. lebbek* backs against experimental Hymenolepis diminuta infection in rats. *Journal of Ethnopharmacology*, *31*, 333-337.

Galle, J., Bengen, J., Schollmeyer and P., Wanner, C. (1995). Impairment of endothelium-dependent dilation in rabbit renal arteries by oxidized lipoprotein (a): role of oxygen-derived radicals. *Circulation*, *92*, 1582-1589.

Garg, A.K., Buchholz, T.A. and Aggarwal, B.B. (2005). Chemosensitization and radiosensitization of tumours by plant polyphenols. *Antioxidant and Redox Signaling*, *7* (11-12), 1630-1647.

Gate, L., Paul, J., Ba,G.N., Tew, K.D. and Tapiero, H. (1999). Oxidative stress induced in pathologies: The role of antioxidants. *Biomedicine and Pharmacotherapy*, *53* (4), 169-180.

Gavrieli, Y., Sherman, Y. and Ben-Ssasson, S.A. (1992). Identification of programmed cell death *in situ via* specific labeling of nuclear DNA fragmentation. *Journal of Cell Biology*, *119*, 493-501.

Geissman, T.A. and Crout, D.H.G. (1969). *Organic chemistry of secondary plant metabolism.* United States of America: Freeman, Cooper & Company.

Geran, R.I., Greenberg, N.H., McDonald, M.M., Schumacher, A.M., and Abbott, B.J. (1997). Modified protocol for the testing of new synthetics in the L1210 lymphoid leukemia murine model in the DR&D program, DCT, NCI. *National Cancer Institute Monographs*, *45*, 151-153.

Geran, R.I., Greenberg, N.H., McDonald, M.M., Schumacher, A.M., and Abbott, B.J. (1972). Protocols for screening chemical agents and natural products against animal tumour and other biological systems. *Cancer Chemotherapy Reports*, *3* (2), 17-19.

Gille, L., Kleiter, M., Willmann, M. and Nohl, H. (2002). Paramagnetic species in the plasma of dogs with lymphoma prior to and after treatment with doxorubicin: An ESR study. *Biochemistry Pharmacology*, *64*, 1737-1744.

Ginzinger, D.G. (2002). Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. *Experimental Hematology*, *30*, 503-512.

Girard, M.P., Steele, D., Chaignat, C. and Kieny, M.P. (2006). A review of vaccine research and development: human enteric infections. *Vaccine*, *24*, 2732-2750.

Goh, K.L. (2000). *Malaysian Herbaceous Plants* (millennium ed.). Malaysia: Advanco Press. (in Chinese).

Goh, S.H., Soepadmo and Chuah, C.H. (1993). *Phytochemical guide to Malaysian flora* (2nd ed.). Kuala Lumpur: Institute of Advanced Studies, University Malaya Press.

Goidin, D., Mamessier, A., Staquet, M.-J., Schmitt, D. and Odile, B.-V. (2001). Ribosomal 18S RNA prevails over Glyceraldehyde-3-Phosphate Dehydrogenase and β-actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. *Analytical Biochemistry, 295* (1), 17-21.

Gorczyca, W., Bigman, K., Mittelman,A., Ahmed, T., Gong, J., Melamed, M.R. and Darzynkiewicz, Z. (1993). Induction of DNA strand breaks associated with apoptosis during treatment of leukemias. *Leukemia, 7*, 659-670.

Grasl-Kraup, B., Ruttkay-Nedecky, B. and Koudelka, H. (1995). *In situ* detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis and autolytic cell death: A cautionary note. *Hepatology, 21*, 1465.

Greenwald, P., Nixon, D.W., Malone, W.F., Kelloff, G.J., Stern, H.R. and Witkin, K.M. (1990). Concepts in cancer chemoprevention research. *Cancer, 65* (5-8), 1483-1490.

Gülçin, I., Uğuz, M.T., Oktay, M., Beydemir, Ş. and Küfrevioğlu, Ö.İ. (2003). Antioxidant and antimicrobial activities of *Teucrium polium* L. *Journal of Food Technology*, *1*, 9–17.

Gülçin, İlhami, Küfrevioğlu, Ö. İrfan, Oktay, Münir and Büyükokuroğlu, Mehmet Emin. (2004). Antioxidant, antimicrobial, antiulcer and analgesic activities of nettle (*Urtica dioica* L.). *Journal of Ethnopharmacology*, *90*, 205-215.

Gupta, M.P., Monge, A., Karikas, G.A., Lopez de Cerain, A., Solis, P.N., De Leon, E., Trujillo, M., Suarez, O., Wilson, F., Montenegro, G., Noriega, Y., Santana, A.I., Correa, M. and Sanchez, C. (1996). Screening of Panamanian medicinal plants for brine shrimp toxicity, crown gall tumour inhibition, cytotoxicity and DNA intercalation. *International Journal of Pharmacognosy*, *34*, 18-27.

Gutierrez, R.M.P. (2006). Identification of triterpenoids in chloroform extract of *Agarista Mexicana* by MS and NMR. *Natural Product Research, 20*, 181-185.

Halliwell, B. and Gutteridge, J.M.C. (1999). *Free radicals in biology and Medicine* (3rd ed.). London: Oxford University Press.

Halliwell, B., Gutteridge, J.M.C. and Cross, C.E. (1992). Free radicals, antioxidants and human disease: where are we now? *Journal of Laboratory and Clinical Medicine*, *119* (6), 598-620.

Hao, J., Zhang, B., Liu, B., Lee, M., Hao, X., Reuhl, K.R., Chen, X. and Yang, C.S. (2009). Effect of α-tocopherol, N-acetylcysteine and omeprazole on esophageal adenocarcinoma formation in a rat surgical model. *International Journal of Cancer*, *124*, 1270-1275.

Harborne, J. (2001). Twenty-five years of chemical ecology. *Natural Products Reports*, *18*, 361-379.

Harvey, A.L. (1999). Medicines from nature: Are natural products still relevant to drug discovery? *Trends in Pharmacological Sciences*, *20* (5), 196-198.

Havrilesky, L.J., Elbendary, A., Hurteau, J.A., Whitaker, R.S., Rodriguez, G.C. and Berchuck, A. (1995). Chemotherapy-induced apoptosis in epithelial ovarian cancers. *Obstetric and Gyanecology*, *85*, 1007-1010.

Heid, C.A., Stevens, J., Livak, K.J. and Williams, P.M. (1996). Real time quantitative PCR. *Genome Research*, *6* (10), 986-994.

Hibasami, H., Kyohkon, M., Ohwaki, S., Katsuzaki, H., Imai, K., Ohnishi, K., Ina, K. and Komiya, T. (2002). Diol- and triol-types of phytol induce apoptosis in lymphoid leukemia molt 4B cells. *International Journal of Molecular Medicine*, *10* (5), 555-559.

Higuchi, R., Dollinger, G., Walsh, P.S. and Griffith, R. (1992). Simultaneous amplification and detection of specific DNA sequences. *Nature Biotechnology*, *10*, 413-417.

Higuchi, R., Fockler, C., Dollinger, G. and Watson, R. (1993). Kinetic PCR analysis: Real-time monitoring of DNA amplification reactions. *Biotechnology*, *11*, 1026-1030.

Hirota, A., Taki, S., Kawaii, S., Yano, M. and Abe, N. (2000). 1,1-Diphenyl-2-picrylhydrazyl radical-scavenging compounds from soyabean miso and antiproliferative activity of isoflavones from soyabean miso toward the cancer cell lines. *Bioscience, Biotechnology and Biochemistry*, *64*, 1038-1040.

Hod, Y. (1992). A simplified ribonuclease protection assay. *Biotechniques*, *13*, 852-854.

Hoffmann, J.J., Timmerman, N., Mclaughlin, R. and Punnapayak, H. (1993). Potential antimicrobial activity of plants from the south western United States. *International Journal of Pharmacology*, *31*, 101-115.

Homma, Y., Ikeda, I., Ishikawa, T., Tateno, M., Sugano, M. and Nakamura, H. (2003). A randomized, placebo-controlled trial: Decrease in plasma low-density lipoprotein cholesterol, apolipoprotein B, cholesteryl ester transfer protein, and oxidized low-density lipoprotein by plant stanol ester-containing spread. *Nutrition*, *19*, 369-374.

Hong, M.Y., Chapkin, R.S., Davidson, L.A., Turner, N.D., Morris, J.S., Carroll, R.J. and Lupton, J.R. (2003). Fish oil enhances targeted apoptosis during colon tumour initiation in part by downregulating Bcl-2. *Nutrition and Cancer*, *46*, 44-51.

Houghton, P.J. and Raman, A. (1998). *Laboratory handbook for the fractionation of natural extracts*. UK: Chapman & Hall.

http://bioweb.uwlax.edu/, 8 April 2009.

http://davesgarden.com/pf/go/57249/index.html, 26 June 2006

http://en.wikipedia.org/wiki/Cactaceae, 20 July 2008.

http://en.wikipedia.org/wiki/Gene_expression, 20 April 2009.

http://en.wikipedia.org/wiki/Pereskia, 16 April 2006.

http://herb4cancer.files.wordpress.com/2007/11/cell-cycle2.jpg, 19 August 2009.

http://toptropicals.com/cgi-bin/garden_catalog/cat.cgi?uid=pereskia_bleo,16 April 2006.

http://www.biologie.uni-hamburg.de/b-online/vascular/cact.htm, 06 July 2006.

http://www.desert-tropicals.com/Plants/Cactaceae/Pereskia_bleo.html, 07 July 2006.

http://www.doacs.state.fl.us/pi/enpp/94-7&8all.htm, 07 July 2006.

http://www.hear.org/gcw/html/autogend/species/14349.HTM, 07 July 2006.

http:// www.invitrogen.com/lux, 20 April 2009.

http://www.plantoftheweek.org/week219.shtml, 16 April 2006.

Huang, D., Ou, B. and Prior, R.L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry, 53*, 1841-1856.

Huang, L.F., Zhong, K.J., Sun, X.J., Wu, M.J., Huang, K.L., Liang, Y.Z., Guo, F.Q. and Li, Y.W. (2006). Comparative analysis of the volatile components in cut tobacco from different locations with gas chromatography-mass spectrometry (GC-MS) and combined chemometric methods. *Analytica Chimica Acta*, *575*, 236-245.

Huerta, S., Goulet, E.J. and Huerta-Yepez, S. (2007). Screening and detection of apoptosis. *Journal of Surgical Research, 139*, 143-156.

Huschtscha, L.J., Bartier, W.A., Ross, C.E.A. and Tattersall, M.H. (1996). Characteristics in cancer deateh after exposure to cytotoxic drugs *in vitro*. *British Journal of Cancer, 73*, 54-60.

Huxley, A. J. (1992)**.** *New Royal Horticultural Society dictionary of gardening* (pp. 240). London: MacMillan Press.

Ichimura, E., Fukuda, T. and Oyama, T. (1995). Formalin fixation by boiling: Is it suitable for the TUNEL staining? *Pathology International, 45*, 971.

Jackson, S.J., Houghton, P.J., Retsas, S. and Photiou, A. (2000). *In vitro* cytotoxicty of norviburtinal and isopinnatal from *Kigelia pinnata* against cancer cell lines. *Planta Medica*, *66*, 758.

Janicke, R.U., Sprengart, M.L., Wati, M.R. and Porter, A.G. (1998). Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *The Journal of Biological Chemistry, 273*, 9357-9360.

Jayaprakasha, G.K., Singh, R.P. and Sakariah, K.K. (2001). Antioxidant activity of grape seed (*Vitis vinifera*) extracts on peroxidation models *in vitro*. *Food Chemistry, 73*, 285-290.

Jiang, Q.,Christen, S., Shigenaga, M.K. and Ames, B.N. (2001). ɤ-Tocopherol, the major form of vitamin E in the US diet, deserves more attention. *American Journal of Clinical Nutrition, 74*, 714-722.

Ju, Y.H., Clausen, L.M., Allred, K.F., Almada, A.L. and Helferich, W.G. (2004). ß-Sitosterol, ß-sitosterol glucoside, and a mixture of ß-sitosterol and ß-sitosterol glucoside modulate the growth of estrogen-responsive breast cancer cells *in vitro* and in ovariectomized athymic mice. *Journal of Nutrition, 134*, 1145-1151.

Kaabinejadian, S., Fouladdel, Sh., Ramezani, M. and Azizi, E. (2008). p53 expression in MCF7, T47D and MDA-MB 468 breast cancer cell lines treated with adriamycin using RT-PCR and Immunocytochemistry. *Journal of Biological Sciences, 8* (2), 380-385.

Kadoma, Y., Ito, S., Atsumi, T. and Fujisawa, S. (2009). Mechanisms of cytotoxicity of 2- or 2,6-di-tert-butylphenols and 2-methoxyphenols in terms of inhibition rate constant and a theoretical parameter. *Chemosphere, 74*, 626-632.

Kamesaki, H. (1998). Mechanisms involved in chemotherapy-induced apoptosis and their implications in cancer chemotherapy. *International Journal of Hematology, 68*, 29-43.

Kamitori, Yasuhiro, Hojo, Masaru, Masuda, Ryoichi, Izumi, Tatsuo and Tsukamoto, Shuichi. (1984). Silica gel as an effective catalyst for the alkylation of phenols and some heterocyclic aromatic compounds. *Journal of Organic Chemistry, 49*, 4161-4165.

Kampa, M., Hatzoglou, A., Notas, G., Damianaki, A., Bakogeorgou, E. and Gemetzi, C. (2000). Wine antioxidant polyphenols inhibit the proliferation of human prostate cancer cell lines. *Nutrition and Cancer, 37*, 223-233.

Karmali, M.A. (2004). Infection by Shiga toxin-producing *Escherichia coli*: An overview. *Molecular Biotechnology, 26*, 117-122.

Kaufman, S.H. (1989). Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin and other cytotoxic anticancer drugs: A cautionery note. *Cancer Research, 49*, 5870-5878.

Kehidupan Sihat. (2006). Vol. 56. Kuala Lumpur: G.G. Enterprise. (in Chinese).

Kellogg, D.E., Sninsky, J.J. and Kowk, S. (1990). Quantitation of HIV-1 proviral DNA relative to cellular DNA by the polymerase chain reaction. *Analytical Biochemistry, 189*, 202-208.

Kerr, J.F., Wyllie, A.H. and Currie, A.R. (1972). Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer, 26*, 239-257.

Kerr, J.F.R., Winterford, C.M. and Harmon, B.V. (1994). Apoptosis-its significance in cancer and cancer therapy. *Cancer, 73*, 2013-2026.

Khanna, C., Lund, E.M., Redic, K.A., Hayden, D.W., Bell, F.W., Goulland, E.L. and Klausner, J.S. (1998). Randomized controlled trial of doxorubicin versus dactinomycin

in a multiagent protocol for treatment of dogs with malignant lymphoma. *Journal of the American Veterinary Medical Association, 213*, 985-990.

Kimura, K., Sasano, H., Shimosegawa, T., Kato, K., Noguchi, T., Mochizuki, S., Sawai, T., Koizumi, M., Toyota, T. and Nagura, H. (1997). Ultrastructural and confocal laser scanning microscopic examination of TUNEL-positive cells. *Journal of Pathology*, *181*, 135-142.

Kirsh, V.A., Mayne, S.T., Peters, U., Chatterjee, N., Leitzmann, M.F. and Dixon, L.B. (2006). A prospective study of lycopene and tomato product intake and risk of prostate cancer. *Cancer Epidemiology Biomarkers and Prevention*, *15*, 92-98.

Kleinsmith, L.J. (2006). *Principles of cancer biology*. USA: Pearson education.

Kline, K., Yu, W. and Sanders, B.G. (2001). Vitamin E: Mechanisms of action as tumour cell growth inhibitors. *Journal of Nutrition*, *131* (1), 161S-163S.

Knox, P., Uphill, P.F., Fry, J.R., Benford, J. and Balls, M. (1986). The FRAME multicentre project on *in vitro* cytotoxicology. *Food and Chemical Toxicology*, *24*, 457-463.

Kogure, K., Manabe, S., Suzuki, I., Tokumura, A. and Fukuzawa, K. (2005). Cytotoxicity of α-tocopheryl succinate, malonate and oxalate in normal and cancer cells *in vitro* and their anti-cancer effects on mouse melanoma *in vivo*. *Journal of Nutritional Science and Vitaminology*, *51*, 392-397.

Kola, I. and Landis, J. (2004). Can the pharmaceutical industry reduce attrition rates? *Nature Reviews Drug Discovery*, *3*, 711-716.

Komiya, T., Kyohkon, M., Ohwaki, S., Eto, J., Katsuzaki, H., Imai, K., Kataoka, T., Yoshioka, K., Ishii, Y. and Hibasami, H. (1999). Phytol induces programmed cell death in human lymphoid leukemia Molt 4B cells. *International Journal of Molecular Medicine, 4*, 377-380.

Kong, A.-N. T., Yu, R., Hebbar, V., Chen, C., Owuor, E., Hu, R., Ee, R. and Mandlekar, S. (2001). Signal transduction events elicited by cancer prevention compounds. *Mutation Research, 480-481*, 231-241.

Kothakota, S., Azuma, T., Reinhard, C., Klippel, A., tang, J., Chu, K., McGarry, T.J., Kirschner, M.W., Koths, K., Kwiatkowski, D.J. and Williams, L.T. (1997). Caspase-3 generated fragment of gelsolin effector of morphological change in apoptosis. *Science*, *278*, 294-298.

Kozubek, A. and Tyman, J.H. (1999). Resorcinolic lipids, the natural non-isoprenoid phenolic amphiphiles and their biological activity. *Chemical Reviews*, *99*, 1-26.

Kuida, K., Zheng, T.S., Na, S.Q., Kuan, C.Y., Yang, D., Karasuyama, H., Rakic, P. and Flavell, R.A. (1996). Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature, 383*, 368-372.

Kulisic, T., Radonic, A., Katalinic, V. and Milos, M. (2004). Use of different methods for testing antioxidative activity of oregano essential oil. *Food Chemistry*, *83*, 633-640.

Kumaran, A. and Joel karunakaram, R. (2006). Antioxidant and free radical scavenging activity of an aqueous extract of Coleus aromaticus. *Food Chemistry*, *97*, 109-114.

Lacroix, M., Toillon, R.A. and Leclercq, G. (2006). p53 and breast cancer, an update. *Endocrine-Related Cancer, 13* (2), 293-325.

Lamson, D.W. and Brignall, M.S. (1999). Antioxidants in cancer therapy: their actions and interactions with oncologic therapies. *Alternative Medicine Review, 4*, 30-329.

Lane, D.P. (1992). P53, guardian of the genome. *Nature, 358*: 15.

Larson, R.A. (1988). The antioxidant of higher plants. *Phytochemistry, 27* (4), 969-978.

Lee, C.C. and Houghton, P. (2005). Cytotoxicity of plants from Malaysia and Thailand used traditionally to treat cancer. *Journal of Ethnopharmacology, 100*, 237-243.

Lee, H.L. and Halliwell, B. (2001). Antioxidant and prooxidant abilities of foods and beverages. *Methods in Enymology*, *335*, 181-190.

Lee, K.-I., Rhee, S.-H. and Park, K.-Y. (1999). Anticancer activity of phytol and eicosatrienoic acid identified from *Perilla* leaves. *Han'guk Sikp'um Yongyang Kwahak Hoechi, 28* (5), 1107-1112. (Journal written in Korean). Retrieved from ScienceFinder.

Levison, W.E. and Jawetz, E. (1992). *Medical Microbiology and Immunology* (2nd ed.). New York: Appleton and Lange.

Li, Y., Sharov, V.G., Jiang, N., Zaloga, C., Sabbah, H.N. and Chopp, M. (1995). Ultrastructural and light microscopic evidence of apoptosis after middle cerebral artery occlusion in the rat. *American Journal of Pathology*, *146*, 1045-1051.

Liang, Y., Lin-Shiau, S., Chen, C. and Lin, J. (1999). Inhibition of cyclin-dependant kinase 2 and 4 activities as well as induction of CDK inhibitors p21and p27 during growth arrest of human breast carcinoma cells by (-) epigallocatechin-3-gallate. *Journal of Cellular Biochemistry*, *75*, 1-12.

Liebsch, H.M. and Spielmann, H. (1995). Balb/c 3T3 cytotoxicity test. In S. O'Hare and C.K. Atterwill (Eds.), *In vitro toxicity testing protocols* (pp.177-187). US: Human Press Inc.

Lih, S.L., Su, T.C. and Wen, W.C. (2001). Studies on the antioxidative activities of Hsian tsao (*Mesona procumbens* Hemsl) Leaf Gum. *Journal of Agricultural Food Chemistry*, *49*, 963-968.

Lin, C., Jensen, K.L. and Yen, S.T. (2005). Awareness of foodborne pathogens among US consumers. *Food quality and Preference, 16*, 401-412.

Liu, S., Manson, J.E., Lee, I.M., Cole, S.R., Hennekens, C.H., Willett, W.C. and Buring, J.E. (2000). Fruits and vegetables intake and risk of cardiovascular disease: The Women's Health Study. *American Journal of Clinical Nutrition, 72*, 922-928.

Liu, X., Zou, H., Slaughter, C. and Wang, X. (1997). DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell*, *89*, 175-184.

Lowe, B., Avila, H.A., Bloom, F.R., Gleeson, M. and Kusser, W. (2003). Quantitation of gene expression in neural precursor by reverse-transcription polymerase chain reaction using self-quenched, fluorogenic primers. *Analytical Biochemistry*, *315*, 95-105.

Lucassen, P.J., Chung, W.C. and Vermeulen, J.P. (1995). Microwave-enhanced in situ end-labeling of fragmented DNA: Parametric studies in relation to postmortem delay and fixation of rat and human brain. *Journal of Histochemistry and Cytochemistry*, *43*, 1163.

Matsuse, I.T., Lim, Y.A., Hattori, M., Correa, M. and Gupta, M.P. (1999). A search for anti-viral properties in Panamanian medicinal plants: The effects on HIV and its essential enzymes. *Journal of Ethnopharmacology*, *64*, 15-22.

Mau, J.L., Lin, H.C. and Song, S.F. (2002). Antioxidant properties of several specialty mushrooms. *Food Research International, 35*, 519-526.

May, P. and May, E. (1999). Twenty years of p53 research: Structural and functional aspects of the p53 protein. *Oncogene*, *18*, 7621-7636.

McCarthy, J.V., Fernandes, R.S. and Cotter, T.G. (1994). Increased resistance to apoptosis associated with HL-60 myeloid differentiation status. *Anticancer Research*, *14*, 2063-2072.

McCormick, C.C. and Parker, R.S. (2004). The cytotoxicity of Vitamin E is both vitamer- and cell-specific and involves a selectable trait. *The Journal of Nutrition*, 3335-3342.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M. and Tauxe, R.V. (1999). Food-related illness and death in the United States. *Emerging Infectious Diseases*, *5*, 607-625.

Meek, D.W. (2004). The p53 response to DNA damage. *DNA Repair*, *3* (8-9), 1049-1056.

Melnikova, V., Bezdetnaya, L., Belitchenko, I., Potapenko, A., Merlin, J.L. and Guillemin, F. (1999). Meta-tetra (hydroxyphenyl)-chlorine-sensitized photodynamic damage of cultured tumour and normal cells in the presence of high concentrations of alpha-tocopherol. *Cancer Letter*, *139* (1), 89-95.

Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.K. (1984). Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Research, 17*, 7035-7056.

Meyn, R.E., Stephens, L.C., Hunter, N.R. and Milas, L. (1995). Apoptosis in murine tumours treated with chemotherapy agents. *Anticancer Drugs*, *6*, 443-450.

Michalak, E., Villunger, A., Erlacher, M. and Strasser, A. (2005). Death squads enlisted by the tumour suppressor p53. *Biochemical and Biophysical Research Communications, 331* (3), 786-798.

Mills, A.A. (2006). P53: oncogene or tumour suppressor? *Genes Development, 16,* 38-44.

Ming, D.S., Tian, Y.A., Li, H.H. and Zhong, L.C. (2004). Diterpene derivative and chromone from *Hypericum perforatum*. *Natural Product Research, 18* (1), 15-19.

Mitova, M., Taskova, R., Popov, S., Berger, R.G., Krings, U. and Handjieva, N. (2003). GC/MS analysis of some bioactive constituents from *Carthamus lanatus* L.Z. *Naturforsch, 58c*, 697-703.

Moghadasian, M.H. (2000). Pharmacological properties of plant sterols: *in vivo* and *in vitro* observations. *Life Science, 67*, 605-615.

Moghadasian, M.H., McManus, B.M., Godin, D.V., Rodrigues, B. and Frohlich, J.J. (1999). Proatherogenic and antiatherogenic effects of probucol and phytosterols in apolipoprotein E-deficient mice: Possible mechanisms of action. *Circulation, 99*, 11733-1739.

Moghadasian, M.H., McManus, B.M., Pritchard, P.H. and Frohlich, J.J. (1997). "Tall oil"- derived phytosterols reduce atherosclerosis in ApoE-deficient mice. *Arteriosclerosis, Thrombosis and Vascular Biology, 17*, 119-126.

Moreau, R., Whitaker, B. and Hicks, K. (2002). Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. *Progress in Lipid Research, 41,* 457.

Moreira, L.F., Naomoto, Y., Hamada, M., Kamikawa, Y. and Orita, K. (1995). Assessment of apoptosis in eosophageal carcinoma preoperatively treated by chemotherapy and radiotherapy. *Anticancer Research, 15*, 639-644.

Morrison, T.B., Weis, J.J. and Wittwer, C.T. (1998). Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *Biotechniques*, *24*, 954-962.

National Committee for Clinical Laboratory Standards (2005). *Performance standards for antimicrobial susceptibility testing 2005* (15th ed.). Wayne Publishers, PA: CLSI/NCCLS.

Nazarenko, I., Pires, R., Lowe, B., Obaidy, M. and Rashtchian. (2002a). Effect of primary and secondary structure of oligodeoxyribonucleotides on the fluorescent properties of conjugated dyes. *Nucleic Acids Research, 30* (9), 2089-2195.

Nazarenko, I., Lowe, B., Darfler, M., Ikonomi, P., Schuster, D. and Rashtchian, A. (2002b). Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore. *Nucleic Acids Research, 30* (9), e37.

Nes, W., David, N., Robert A. and Benson, M. (1992). Carbon-13 NMR studies on sitosterol biosynthesized from [^{13}C] mevalonates. *Phytochemistry, 31* (3), 805-811.

Newman, D.J., Gragg, G.M. and Snader, K.M. (2003). Natural products as sources of new drugs over the period 1981-2002. *Journal of Natural Products, 66*.

NIST 05 MS Library. (2002). Palo Alto, CA, USA: Agilent Technologies.

Noble, W.C. (1998). Skin bacteriology and the role of *Staphylococcus aureus* in infection. *British Journal of Dermatology, 139*, 9-12.

Nogueira, P.C. de L., Bittrich, V., Shepherd, G.J., Lopes, A.V. and Marsaiolia, A.J. (2001). The ecological and taxonomic importance of flower volatiles of *Clusia* species (Guttiferae). *Phytochemistry, 56*, 443-452.

Nolan, T., Hands, R.E. and Bustin, S.A. (2006). Quantification of mRNA using real-time RT-PCR. *Nature Protocols, 1* (3), 1559-1582.

Nunez, G., Benedict, M.A., Hu, Y. and Inohara, N. (1998). Caspases: The proteases of the apoptotic pathway. *Oncogene, 17*, 3237-3245.

O'Brien, M., Okragly, A., Warner, T., Kalil, R. and Wolswijk, G. (1998). DeadEnd™ Colorimetric TUNEL System: Application in Pathology. *Promega Notes, 69*, 2.

OECD (Organization). (1998). *Harmonized integrated hazard classification system for human health and environmental effects of chemical substances.* Paris: OECD.

Ohno, T., Inoue, M. and Ogihara, Y. (2001). Cytotoxic activity of gallic acid against liver metastasis of mastocytoma cells P-815. *Anticancer Research, 21*, 3875-3880.

Oliver, J.A. (1986). Opportunities for using fewer animals in acute toxicity studies. In *Chemicals testing and animal welfare* (pp. 119-142). Solna, Sweden: The National Chemicals Inspectorate.

Olson, H., Betton, G., Robinson, D., Thomas, K., Monro, A., Kolaja, G., Lilly, P., Sanders, J., Sipes, G., Bracken, W., Dorato, M., Deun, K.V., Smith, P. and Berger, B., Heller, A. (2000). Concordance of the toxicity of pharmaceuticals in humans and in animals. *Regulatory Toxicology and Pharmacology, 32*, 56-67.

Ooi, K.L., T.S. Tengku Muhammad and S.F. Sulaiman. (2003). Cytotoxic effect of *Pereskia grandifolia* extracts against different classes of cell lines. Proceedings of the 14th National Biotechnology Seminar, USM Penang, Malaysia.

Osawa, T. (1995). Plant antioxidants: Discusses the potential protective role of botanically derived molecules against oxygen radical species. *Skin Care Applied Research Series*, 27-31.

Osborne, C.K., Hobbs, K. and Trent, J.M. (1987). Biological differences among MCF-7 human breast cancer cell lines from different laboratories. *Breast Cancer Research and Treatment, 9*, 111-121.

Oyaizu, M. (1986). Studies on products of browning reactions: Antioxidative activities of products of browning reaction prepared from glucosamine. *Japanese Journal of Nutrition, 44*, 307-315.

Parker, R.M. and Barnes, N.M. (1999). mRNA: Detection by *in situ* and northen hybridization. *Methods in Molecular Biology*, *106*, 247-283.

Pereira, A.S., Siqueira, D.S., Elias, V.O., Simoneit, B.R.T., Cabral, J.A. and Neto, F.R.A. (2002). Three series of high molecular weight alkanoates found in Amazonian plants. *Phytochemistry*, *61*, 711-719.

Pettit, G.R. (1997). *Biosynthetic products for cancer chemotherapy*. New York: Plenum Press.

Phutdhawong, W., Donchai, A., Korth, J., Pyne, S.G., Picha, P., Ngamkham, J. and Buddhasukh, D. (2004). The components and anticancer activity of the volatile oil from *Streblus asper*. *Flavour and Fragrance Journal*, *19* (5), 445-447. Abstracted from ScienceFinder.

Pillai, G.R., Srivastava, A.S., Hassanein, T.I., Chauhan, D.P. and Carrier, E. (2004). Induction of apoptosis in human lung cancer cells by curcumin. *Cancer Letter*, *208*, 163-170.

Plachta, H., Bartnikowska, E. and Obara, A. (1992). Lipid peroxides in blood from patients with atherosclerosis of coronary and peripheral arteries. *Clinical Chimica Acta*, *211*, 101-115.

Pokorny, J. (1991). Natural antioxidants for food use. *Trends in Food Science and Technology*, *2*, 223-227.

Polverino, A.J. and Patterson, S.D. (1997). Selective activation of caspases during apoptotic induction in HL-60 cells. *Journal of Biological Chemistry, 272*, 7013-7021.

Pratt, D.E. (1992). Natural antioxidants from plant material. In M.T. Huang, C.-T. Ho and C.Y. Lee (Eds.), *Phenolic compounds in food and their effects on health II. Antioxidants and cancer prevention, ACS Symposium Series 507* (pp. 54-71). Washington: American Chemical Society.

Prendergast, G.C. (1999). Mechanisms of apoptosis by c-Myc. *Oncogene*, *18*, 2967-2987.

Prior, R.L., Wu, X. and Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, *53*, 4290-4302.

Pulido, R., Bravo, L. and Saura-Calixto, F. (2000). Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *Journal of Agricultural and Food Chemistry*, *48*, 3396-3402.

Pulkkanen, K.J., Laukkanen, M.O., Naarala, J. and Yla-Herttuala, S. (2000). False-positive apoptosis signal in mouse kidney and liver detected with TUNEL assay. *Apoptosis*, *5*, 329-333.

Rahman, A., Choudhary, M.I. and Thomsen, W.J. (2001). *Bioassay techniques for drug development*. Netherlands: Harwood Academic Publishers.

Rahmat, A. (2004). Daun jarum tujuh bilah untuk barah, sakit kepala. *Info Herba, Mingguan Malaysia, 5 December.* Malaysia: Utusan Melayu (M) Bhd.

Ramarathnam, N., Osawa, T., Ochi, H. and Kawakishi, S. (1995). The contribution of plant food antioxidants to human health. *Trends in Food Science and Technology*, *6* (3), 75-82.

Ramzi, A.A. and Ulrike, L. (2005). Antimicrobial activity of some medicinal plants of the island Soqotra. *Journal of Ethnopharmacology, 96*, 177-181.

Rana, V.S. and Blazquez, M. A. (2007). Chemical constituents of *Gynura cusimbua* aerial parts. *Journal of Essential Oil Research*, *19*, 21-22.

Randrianarivelo, R., Sarter, S., Odoux, E., Brat, P., Lebrun, M., Romestand, B., Menut, C., Andrianoelisoa, H.S., Raherimandimby, M. and Danthu, P. (2009). Composition and antimicrobial activity of essential oils of *Cinnamosma fragrans*. *Food Chemistry*, *114*, 680-684.

Rappolee, D.A., Mark, D., Banda, M.J. and Werb, Z. (1988). Wound macrophages express TGF-alpha and other growth factors *in vivo* analysis by mRNA phenotyping. *Science*, *241*, 708-712.

Rates, S.M.K. (2001). Plants as source of drugs. *Toxicon*, *39*, 603-613.

Reddy, L., Odhav, B. and Bhoobla, K.D. (2003). Natural Products for cancer prevention: A global perspective. *Pharmacology and Therapeutics*, *99*, 1-13.

Reed, J.C. (2002). Apoptosis-based therapies. *Nature Reviews Drug Discovery*, *1* (2), 111-121.

Rekhviashvili, N., Stevens, G., Scott, L. and Stevens, W. (2006). Fluorogenic LUX primer for quantitation of HIV-1 by real-time RT-PCR. *Molecular Biotechnology*, *32*, 101-109.

Rensburg, S.J., Daniels, W.M., Zyl, J.M. and Taljaard, J.J. (2000). A comparative study of the effects of cholesterol, beta-sitosterol, beta-sitosterol glucoside, dehydroepiandrosterone sulphate and melatonin on *in vitro* lipid peroxidation. *Metabolic Brain Disease*, *15*, 257-265.

Rocha, A.B., Lopes, R.M. and Schwartsmann G. (2001). Natural products in anticancer therapy. *Current Opinion in Pharmacology*, *1*, 364-369.

Romero, I., Paez, A., Ferruelo, A., Lujan, M. and Berenguer, A. (2002). Polyphenols in red wine inhibit the proliferation and induce apoptosis of LNCaP cells. *BJU International*, *89*, 950-954.

Roemer, M.M., Katsen, A., Mestres, P. and Roemer, K. (2001). Resveratrol induces colon tumour cell apoptosis independently of p53 and preceded by epithelial differentiation, mitochondrial proliferation and membrane potential collapse. *International Journal of Cancer*, *94*, 615-622.

Rouach, H., Fataccioli, V., Gentil, M., French, S.W., Morimoto, M. and Nordmann, M. (1997). Effect of chronic ethanol feeding on lipid peroxidation and protein oxidation in relation to liver pathology. *Hepatology, 2*, 351-355.

Rudel, T. and Bokoch, G. (1997). Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK 2. *Science*, *276*, 1571-1574.

Ruegg, T., Calderon, A.I., Queiroz, E.F., Solis, P.N., Marston, A., Rivas, F., Ortega-Barria, E., Hostettmann, K., Gupta, M.P. (2006). 3-Farnesyl-2-hydroxybenzoic acid is a new anti-*Helicobacter pylori* compound from *Piper multiplinervium*. *Journal of Ethnopharmacology, 103*, 461-467.

Russo, A., Cardile, V., Ioannes, A.D. and Garbarino.(2009). Effect of litreol on the viability of human cancer cells. *Chemico-Biological Interactions, 179*, 178-184.

Saccomanno, C.F., Bordonaro, M., Chen, J.S. and Nordstrom, J.L. (1992). A faster ribonuclease protection assay. *Biotechniques, 13*, 846-850.

Sader, H.S., Jones, R.N. and Silva, J.B. (2002). Skin and soft tissue infections in Latin American Medical Centers: Four-year assessment of the pathogen frequency and antimicrobial susceptibility patterns. *Diagnostic Microbiology of Infectious Diseases*, *44*, 281-288.

Sahu, N. P., Banerji, N. and Chakravarti, R. N. (1974). A new saponin of oleanolic acid from *Pereskia grandifolia*. *Phytochemistry, 13*, 529-530.

Saito, M., Atsumi, T., Satoh, K., Ishihara, M., Iwakura, I., Sakagami, H., Yokoe, I. and Fujisawa, S. (2001). Radical production and cytotoxic activity of tert-butyl-substituted phenols. *In vitro and Molecular Toxicology*, **14** (1), 53-63.

Sakahira, H., Enari, M. and Nagata, S. (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature*, *391*, 96-99.

Sakanaka, S., Tachibana, Y., Ishihara, N. and Juneja, L.R. (2004). Antioxidant activity of egg yolk hydrolysates in a linoleic acid oxidation system. *Food Chemistry*. ***86*** (1), 99-103.

Sakan, T., Isoe, S. and Hyeon, S.B. (1967). The structure of actinidiolide, dihydroactinidiolide and actinidol. *Tetrahedron Letter*, *4*, 1623-1627.

Santos, Alfredo C., Guevara, Bweatrice Q., Mascardo, Alicia M. and Estrada, Concepcion. (1978). *Phytochemical, microbiological and pharmacological screening of medicinal plants: A supplement of the Acta Manilana.* Philippines: GMS Publishing Corporation.

Sato, Y., Hotta, N., Sakamoto, N., Matsuko, S., Oshishi, N. and Yagi, K. (1979). Lipid peroxide level in plasma of diabetic patients. *Biochemical Medicine*, *21*, 104-107.

Scheers, M.E., Ekwall, Ba. and Dierickx, J.P. (2001). *In vitro* long-term cytotoxicity testing of 27 MEIC chemicals on HepG2 cells and comparison with acute human toxicity data. *Toxicology in Vitro*, *15*, 153-161.

Schrek, R., Albermann, K. and Baeuerle, A. (1992). Nuclear factor B: an oxidative stress- responsive transcription factor of eukaryotic cells: A review. *Free Radical Research Communications, 17*, 221-237.

Schwartz, J., Shklar, G. and Trickler, D. (1993). p53 in the activation mechanism of vitamin E. *European Journal of Cancer with Cancer Oncology, 29B* (4), 313-318.

Scott, W.L. and Athena W.L. (2000). Apoptosis in cancer. *Carcinogenesis, 21* (3), 485-495.

Service, R.F. (1995). Antibiotics that resist resistance. *Science, 270*, 724-727.

Sharkey, F.H., Banat, I.M. and Marchant, R. (2004). Detection and quantification of gene expression in environmental bacteriology. *Applied and Environmental Microbiology*, **70** (7), 3795-3806.

Shi, Y., Glynn, J.M., Guilbert, L.J., Cotter, T.G., Bissonnette, R.P. and Green, D.R. (1992). Role of c-myc in activation-induced apoptotic cell death in T cell hybridomas. *Science, 257*, 212-214.

Shinomiya, N., Shinomiya, M. and Wakiyama, H. (1994). Enhancement of CDDP cytotoxicity by caffeine is characterized by apoptotic cell death. *Experimental Cell Research, 210*, 236-242.

Shon, M.Y., Kim, T.H. and Sung, N.J. (2003). Antioxidants and free radical scavenging activity of *Phellinus baumii* (*Phellinus* of Hymenochaetaceae) extracts. *Food Chemistry, 82*, 593–597.

Shu, Y.-Z. (1998). Recent natural products based drug development: A pharmaceutical industry perspective. *Journal of Natural Products, 61*, 1053-1071.

Silverstein, R.M. and Webster, F.X. (1998). *Spectrometric identification of organic compounds* (6th ed.). New York: John Wiley & Sons, Inc.

Singleton, V.L. and Rossi, J.A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16*, 144-158.

Sipowicz, M.A., Chomarat, P., Diwan, B.A., Anver, M.A., Awasthy, Y.C. and Ward, J.M. (1997). Increased oxidative DNA damage and hepatocyte overexpression of specific cytochrome P450 isoforms in hepatitis of mice infected with *Helicobacter hepaticus*. *American Journal of Pathology, 4*, 933-941.

Solinas, A., Brown, L.J., McKeen, C., Mellor, J.M., Nicol, J., Thelwell, N. and Brown, T. (2001). Duplex scorpion primer in SNP analysis and FRET applications. *Nucleic Acids Research, 29* (20), E96.

Stadelmann, C., Bruck, W., Bancher, C., Jellinger, K. and Lassmann, H. (1998). Alzheimer disease: DNA fragmentation indicates increased neuronal vulnerability, but not apoptosis. *Journal of Neuropathology and Experimental Neurology, 57*, 456-464.

Stadtman, J.A. (1992). Protein oxidation and aging. *Science, 257*, 1220-1224.

Stallard, N. (2006). Optimal adaptive designs for acute oral toxicity assessment. *Journal of Statistical Planning and Inference*, *136*, 1781-1799.

Stark, D.M., Shopsis, C., Borenfreund, E. and Babich, H. (1986). Prigress and problems in evaluating and validating alternative assays in toxicology. *Food and Chemical Toxicology*, *24*, 449-455.

Steele, V.E., Moon, R.C., Lubet, R.A., Grubbd, C.J., Reddy, B.S., Wargovich, M., McCormick, D.L., Pereira, M.A., Crowell, J.A. and Bagheri, D. (1994). Preclinical efficacy evaluation of potential chemopreventive agents in animal carcinogenesis models: Methods and results from the NCI Chemoprevention Drug Development Program. *Journal of Cellular Biochemistry Supplement*, *20*, 32-54.

Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C. and Witztum, J.L. (1989). Beyond cholesterol: Modifications of low-density lipoprotein that increase its artherogenecity. *The New England Journal of Medicine*, *320*, 915-924.

Steller, H. (1995). Mechanisms and genes of cellular suicide. *Science*, *267*, 1445-1449.

Stocker, R. (1999). Dietary and pharmacological antioxidants in atherosclerosis. *Current Opinion in Lipidology*, *10*, 589-597.

Sun, T. and Ho, C.T. (2005). Antioxidant activities of buckwheat extracts. *Food Chemistry*, *90*, 743-749.

Suzuki, T., Higgins, P.J. and Crawford, D.R. (2000). Control selection for RNA quantitation. *Biotechniques*, *29* (2), 332-337.

Swanson, S.M. and Pezzuto, J.M. (1990). Bioscreening technique for cytotoxicity potential and ability to inhibit macromolecule biosynthesis. In E.B. Thompson (Ed.), *Drug bioscreening: Drug evaluation techniques in pharmacology* (pp. 273-297). New York: VCH Publishers.

Tabart, J., Kevers, C., Pincemail, J., Defraigne, J-O and Dommes, J. (2009). Comparative antioxidant capacities of phenolic compounds measured by various tests. *Food Chemistry*, *113*, 1226-1233.

Takeara, R., P.C. Jimenez, D.V. Wilke, M. Odorico de Moraes, C. Pessoa, N. Peporine Lopes, J.L.C. Lopes, T.M. Cruz Lotufo and L.V. Costa-Lotufo. (2008). Antileukemic effects of *Didemnum psammatodes* (Tunicata: Ascidiacea) constituents. *Comparative Biochemistry and physiology Part A*, *151*, 363-369.

Tanaka, T., Makita, H., Ohnishi, M., Mori, H., Satoh, K., Hara, A., Sumida, T., Fukutani, K. and Ogawa, H. (1997). Chemoprevention of 4-nitroquinoline 1-oxide-induced oral carcinogenesis in rats by flavonoids diosmin and hesperidin, each alone and in combination. *Cancer Research*, *57*, 246-252.

Tanaka, T., Kawabata, K., Honjo, S., Kohno, H., Murakami, M. and Shimada, R. (1999). Inhibition of azoxymethane-induced aberrant crypt foci in rats by natural compounds, caffeine, quercetin and morin. *Oncology Reports*, *6*, 1333-1340.

Tan, M. L., Sulaiman, S. F., Najimuddin, N., Samian, M. R. and Tengku Muhammad, T. S. (2005). Methanolic extract of *Pereskia bleo* (Kunth) DC. (Cactaceae) induces apoptosis in breast carcinoma, T47-D cell line. *Journal of Ethnopharmacology*, *96*, 287-294.

Technical Bulletin of the DeadEnd™ Colorimetric TUNEL System #TB199. (2005). USA: Promega Corporation.

Terry, C.F., Shanahan, D.J., Ballam, L.D., Harris, N., McDowell, D.G. and Parkes, H.C. (2002). Real-time detection of genetically modified soya using lightcycler and ABI 7700 platforms with TaqMan, Scorpion and SYBR Green I chemistries. *Journal of AOAC International*, *8* (4), 938-944.

The Second Report of National Cancer Registry. (2003). Cancer Incidence in Malaysia 2003, 13th September 2004.

Thomas, A.S., Joel, E.T. and John, H.A. (1987). Dominance of Δ5-sterols in eight species of the Cactaceae. *Photochemistry*, *26*, 731-733.

Timbrell, J.A. (2000). Toxic responses to foreign compounds. In J.A. Timbrell (Ed.), *Principles of biochemical toxicology* (3rd ed.) (pp. 272-273). London: Taylor & Francis.

Todd, E.C.D. (1989). Preliminary estimates of cost of foodborne disease in Canada and costs to reduce *Salmonellosis*. *Journal of Food Protection*, *52*, 586-594.

Trafny, E.A. (1998). Susceptibility of adherent organisms from Pseudomonas aeruginosa and Staphylococcus aureus strains isolated from burn wounds to antimicrobial agents. *International Journal of Antimicrobial Agents*, *10*, 223-228.

Trichopoulou, A. (1995). Olive oil and breast cancer. *Cancer Causes Control*, *6*, 475-476.

Trilla, A. and Miro, J.M. (1995). Identifying high risk patients for Staphylococcus aureus infections: Skin and soft tissue infections. *Journal of Chemotherapy*, *7*, 37-43.

Turnbull, P.C.B. and Kramer, J.M. (1991). *Bacilllus*. In A. Barlows, W.J. Hausler, K.L. Herrmann, H.D. Isenberg and H.J. Shadomy (Eds.), *Manuals of clinical microbiology* (5th ed) (pp.345-355). American Washington DC: Society of Microbiology.

Uechi, K., S. Matsuyama and T. Suzuki. (2007). Oviposition attractants for *Plodia interpunctella* (Hubner) (Lepidoptera: Pyralidae) in the volatiles of whole wheat flour. Journal of *Stored Products Research*, *43*, 193-201.

Ukelis, U., Kramer, P.-J., Olejniczak, K., Mueller, S.O. (2008). Replacement of *in vivo* acute oral toxicity studies by *in vitro* cytotoxicity methods: Opportunities, limits and regulatory status. *Regulatory Toxicology and Pharmacology*, *51*, 108-118.

Valentão, P., Fernandes, E., Carvalho, F., Andrade, P.B., Seabra, R.M. and Bastos, M.L. (2002). Antioxidative properties of Cardoon (*Cynara cardunculus* L.) infusion against superoxide radical, hydroxyl radical, and hypochlorous acid. *Journal of Agricultural and Food Chemistry*, *50*, 4989–4993.

Velioglu, Y.S., Mazza, G., Gao, L. and Oomah, B.D. (1998). Antioxidant activity and total phenolics on selected fruits, vegetables, and grain products. *Journal of Agricultural and Food Chemistry, 46*, 4113-4117.

Vousden, K.H. and Lu, X. (2002). Live or let die: the cell's response to p53. *Nature Reviews Cancer*, **2** (8), 594-604.

Walczak, H. and Krammer, P.H. (2000). The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. *Experimental Cell Research, 256*, 58.

Walker, P.R., Smith, C. and Youdale, T. (1991). Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes. *Cancer Research, 51*, 1078-1085.

Walum, E. (1998). Acute oral toxicity. *Environmental Health Perspectives, 106* (Suppl 2), 497-503.

Walum, E., Stenberg, K. and Jenssen, D. (1990). *Understanding cell toxicology: Principal and practice*. New York: Ellis H.

Wang, T. and Brown, M.J. (1999). mRNA quantification by real time TaqMan polymerase chain reaction: validation and comparison with RNase protection. *Analytical Biochemistry, 269*, 198-201.

Wang, T., Hicks, K.B. and Moreau, R. (2002). Antioxidant activity of phytosterols, oryzanol, and other phytosterol conjugates. *Journal of American Oil Chemists' Society, 79*, 1201-1206.

Weis, J.H., Tan, S.S., Martin, B.K. and Wittwer, C.T. (1992). Detection of rare mRNAs *via* quantitative RT-PCR. *Trends in Genetics, 8*, 263-264.

Weiss, R.B. (1992). The anthracyclines: Will we ever find a better doxorubicin? *Seminar Oncology, 19*, 670-686.

Wen, L.P., Farhn, J.A., Troise, S., Guan, J.L., Orth, K. and Rosen, G.D. (1997). Cleavage of focal adhesion kinase (FAK) by caspases during apoptosis. *Journal of biological chemistry, 272*, 26056-26061.

Weyermann, J., Lochmann, D. and Zimmer, A. (2005). A practical note on the use of cytotoxicity assays. *International Journal of Pharmaceutics, 288*, 369-376.

White, M.K. and Cinti, C. (2004). A morphological approach to detect apoptosis based on electron microscopy. *Methods in Molecular Biology, 285*, 105.

Willet, W.C., Sacks, S., Trichopoulou, A., Drescher, G., Fero-Luzzi, A. and Helsing, E. (1995). Mediterranean diet pyramid: A cultural model for healthy eating. *American Journal of Clinical Nutrition, 61*, 1402S-1406S.

Wilson, A.P. (1986). *Cytotoxicity and viability assays in animal cell culture: A practical approach.* New York: IRL Press Limited.

Wilson, A.P. (2000). Cytotoxicity and viability assays. In J.R.W. Master (eEd.), *Animal cell culture: A practical approach* (pp. 175-219). UK: Oxford University Press.

Wiseman, H. and Halliwell, B. (1996). Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochemical Journal*, *313*, 17-29.

Wong, K.H., Sabaratnam, V., Abdullah, N., Kuppusamy, U.R. and Naidu, M. (2009). Effects of cultivation techniques and processing on antimaicrobial and antioxidant activities of *Hericium erinaceus* (Bull.:Fr.) Pers. Extracts. *Food Technology and Biotechnology*, *47* (1), 47-55.

Woo, M., Hakem, R., Soengas, M.S., Duncan, G.S. and Shahinian, A. (1998). Essential contribution of caspase-3/CPP32 to apoptosis and its associated nuclear changes. *Genes development*, *12*, 806-819.

Wu, X.-X., Kakehi, Y., Jin, X.-H., Inui, M. and Sugimoto, M. (2009). Induction of apoptosis in human renal cell carcinoma cells by vitamin E succinate in caspase-independent manner. *Urology*, *73*, 193-199.

Wurm, F.M., Gwinn, K.A. and Kingston, R.E. (1986). Inducible overproduction of mousse c-myc protein in mammalian cells. *Proceedings of the National Academy of Science of the United States of America*, *83*, 5414-5418.

Wyllie, A.H., Kerr, J.F. and Currie, A.R. (1980). Cell death: the significance of apoptosis. *International Review of Cytology*, *68*, 251-306.

Wyllie, A.H., Rose, K.A., Morris, R.G., Steel, C.M., Foster, E. and Spandidos, D.A. (1987). Rodent fibroblast tumours expressing human myc and ras genes; growth, metastasis and endogenous oncogene expression. *British Journal of Cancer*, *56*, 251-259.

Yan, X.J., Fang, G.M. and Lou, Q.X. (1999). Studies on free radical scavenging activity in Chinese seaweeds. Part1. Screening results. Chinese *Journal of Oceanology and Limnology*, *17*, 240-246.

Yang, A. and Kerdel, F.A. (2006). Infectious disease update: new anti-microbials. *Seminars in Cutaneous Medicine and Surgery*, *25*, 94-99.

Yasukawa K., Takido, M., Matsumoto, T., Takeuchi, M. and Nakagawa, S. (1991). Sterol and triterpene derivatives from plants inhibit the effects of tumour promoter and sitosterol and betulinic acid inhibit tumour formation in mouse skin two-stage carcinogenesis. *Oncology*, *41*, 72-76.

Yau, Y.H., Ho, B., Tan, N.S., Ng, M.L. and Ding, J.L. (2001). High therapeutic index of factor C sushi peptides: potent antimicrobials against *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, *45*, 2820-2825.

Yoon, M.-A., Jeong, T.-S., Park, D.-S., Xu, M.-Z., Oh, H.-W., Song, K.-B., Lee, W.S. and Park, H.-Y. (2006). Antioxidant effects of quinoline alkaloids and 2,4-di-tert-butylphenol isolated from *Scolopendra subspinipes*. *Biological and Pharmaceutical Bulletin*, *29*, 735-739.

Yoshimitsu, S., Kanako, S., Hiroshi, A., Yoshikazu, K., Akemichi, N., Norio, Y., Katsuhiro, Y., Hiroshi, T., Akio, O. and Hisashi, K. (2006). Uetrotrophic effect of 2,4-di-tert-butylphenol on ovariectomized CD1 mice. *Annual report of Tokyo Metropolitan Institute of Public Health*, *56*, 343-346.

Yoshioka, K., Deng, T., Cavigelli, M. and Karin, M. (1995). Anti-tumour promotion by phenolic antioxidants: inhibition of AP-1 activity through induction of fra expression. *Proceedings of the National Academy of Sciences USA*, *92*, 4972-4976.

Yoshioka, K. Kataoka, T., Hayashi, T., Hasegawa, M., Ishi, Y. and Hibasami, H. (2000). Induction of apoptosis by gallic acid in human stomach cancer KATO III and colon adenocarcinoma COLO 205 cell lines. *Oncology Reports*, *7*, 1221-1223.

Yuan, A., Yu, C-J, Luh, K-T, Chen, W-J, Lin, F-Y, Kuo, S.H., Yang, P.C. (2000). Quantification of VEGF mRNA expression in non-small cell lung cancer using a real-time quantitative reverse transcription-PCR assay and a comparison with quantitative competitive reserve transcription-PCR. *Laboratory Investigation*, *80* (11), 1671-1680.

Yu, R., Tan, T.H. and Kong, A.T. (1997). Butylated hydroxyanisole and its metabolite tert-butylhydroquinone differentially regulate mitogen-activated protein kinases: The role of antioxidative stress in the activation of mitogen activated protein kinases by phenolic antioxidants. *Journal of Biological Chem*istry, *272* (46), 28962-28970.

Zang, Y., Ni, J., Messing, E.M., Chang, E., Yang, C.Y. and Yeh, S. (2002). Vitamin E succinate inhibits the function of androgen receptor and the expression of prostate-specific antigen in prostate cancer cells. *Proceedings of the National Academy of Sciences USA*, *99*, 7408-7413.

Zhu, N. and Wang, Z. (1997). An assay for DNA fragmentation in apoptosis without phenol/chloroform extraction and ethanol precipitation. *Analytical biochemistry*, *246*, 155-158.

APPENDIX

Appendix A

Cell culture techniques

1. Preparation of medium (Medium 199, RPMI 1640 Medium, McCOY'S 5A Medium and Basic Eagle Minimum Essential Medium)

Basic Medium

The medium was prepared by dissolved medium (Medium 199, RPMI 1640 Medium, McCOY'S 5A medium and Eagle Minimum Essential Medium) powder (Sigma) and 2.0 g of sodium bicarbonate ($NaHCO_3$, Merck, Germany) in 1000 ml of distilled water. The pH of the medium was calibrated to pH 7.4 (Hanna Instruments 8417). The medium was then filter sterilized through a 0.22 µm filter membrane (Schleicher & Schuell) into sterile bottles and kept at 4 ℃ for up to four months.

Complete growth medium

Complete growth medium was prepared by using 90 ml of basic medium, supplemented with 10 % foetal bovine serum (FBS, PAA Lab, Austria), 100 µg/ml penicillin or streptomycin (PAA Lab, Austria) and 50 µg/ml of fungizone (PAA Lab, Austria). The medium was filter sterilized using a 0.22 µm filter membrane (Schleicher & Schuell). The colours of the medium were reddish orange and were kept at 4 ℃ for up to two weeks.

Revival medium

Revival medium was prepared as complete growth medium described above, except revival medium was supplemented with 20 % instead of foetal bovine medium.

Cryopreservation medium

Cryopreservation medium was prepared by 50 % Foetal Bovine Serum (FBS, PAA Lab, Austria), 40 % basic culture medium and 10 % dimethylsulfoxide (DMSO) as cryoprotectant.

2. Preparation of solutions

Phosphate Buffered Saline (PBS)

The Phosphate Buffered Saline (PBS) was prepared using 1.52 g sodium phosphate anhydrous ($NaHPO_4$, Merck, Germany), 0.58 g potassium dihydrogen orthophosphate (KH_2PO_4, Merck, Germany) and 8.5 g sodium chloride (NaCl, BDH AnalaR) were dissolved in distilled water and the volume was made up to 1000 ml. The pH of the buffer was adjusted to 7.2 using a pH meter (Hanna Instruments 8417). The buffer was then filtered using a 0.22 µm filter membrane and autoclaved for 15 min at 15psi, 121 ℃.

Trypsin-EDTA

Trysin-EDTA solution was prepared by dissolving 0.25 g trypsin (Amresco, USA) and 0.03 g EDTA (Sigma, USA) in 100 ml of distilled water. The solution was sterilized by filtration using a 0.22 µm filter membrane and stored at -20 ℃.

0.4 % Tryphan Blue

0.4 % Tryphan Blue solution was prepared by dissolving 0.2 g tryphan blue in 50 ml distilled water.

3. Procedure for cell revival

The vial of cells was removed from liquid nitrogen and plunged into a beaker of ice. It was then transferred to a 37 ℃ water bath for quick thawing. The O-ring and cap were kept out of the water when thawing to reduce the possibility of contamination. The vial was removed from the water bath as soon as the contents are thawed, and decontaminated by spraying with 70 % ethanol. The cells were transferred into 1 ml of revival medium (Medium 199, RPMI 1640 Medium, McCOY'S 5A Medium or Eagle Minimum Essential Medium) in a polypropylene tube (Falcon, USA) and spun at 1000 rpm for 5 min. The supernatant was discarded and the pelleted cells were resuspended in 5-7 ml of complete growth medium by gently pipetting and incubated in a 25 ml tissue culture flask (Falcon, USA) at 37 ℃ in a 5 % CO_2 incubator.

4. Procedure for maintenance of cells

KB cells were maintained in Medium 199 (Sigma); CasKi, A549 and MCF7 cells in RPMI 1640 medium (Sigma); HCT 116 in McCOY'S 5A Medium (Sigma) and MRC-5 cells in EMEM (Eagle Minimum Essential Medium) (Sigma). The culture was incubated in a 5 % CO_2 incubator (Shel Lab water-jacketed) kept at 37℃ in a humidified atmosphere. The culture was subcultured every 2 or 3 days and routinely checked under an inverted microscope (IMT-2 Olympus, Japan) for any contamination.

5. Procedure for subcultivation of cells

Adherent cells were attached and formed a single layer in the culture flask. The culture was examined with an inverted microscope to check for any evidence of microbial contamination and to determine if the majority of cells were still attached to

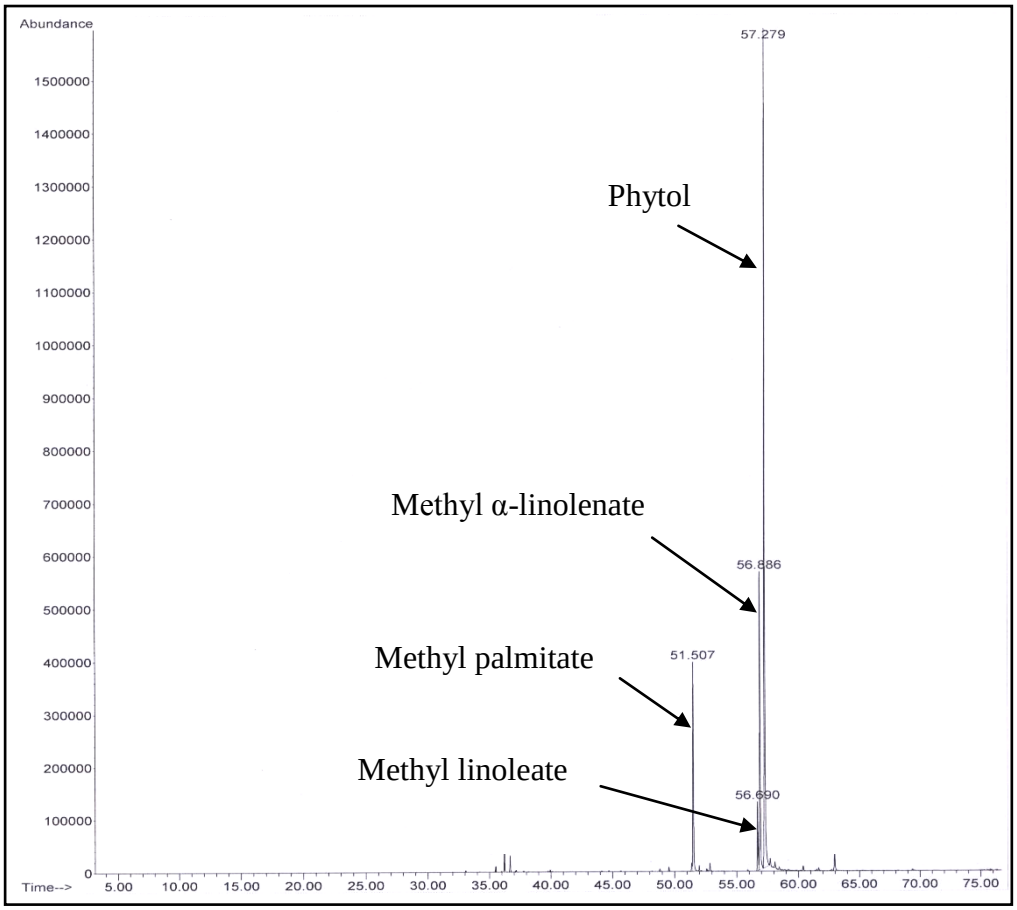
the bottom of the flask. If pH becomes acidic, cells will detach and grow as a suspension that can be transferred by pipettng. If cells were attached, culture medium was discarded. The cell layer was washed twice using 6 to 7 ml of PBS solution to remove all traces of serum. 1 ml of 0.25 % trypsin-EDTA solution and 3 ml of PBS solution were added to the flask and cells were observed under an inverted microscope until the cell layer was dispersed. Cells that were difficult to detach were incubated at 37 ℃ to facilitate dispersal. The entire contents of the flask were transferred aseptically into a centrifuge tube by pipetting. 1 ml of complete growth medium was added and cells were centrifuged for 5 min at 1000 rpm. The supernatant was discarded and pelleted cells were resuspended in 3 to 4 ml of complete growth medium by gently pipetting. Appropriate aliquots of the cell suspension were added to new culture flasks. The flask was then further incubated at 37 ℃ in a 5 % CO_2 incubator.

6. Procedure for cryopreservation of cells

Cell stocks were preserved and stored in liquid nitrogen. Cell suspensions were spun down at 1000 rpm for 5 min using a bench centrifuge (Clements 2000) after the medium was discarded. The supernatant was discarded and pelleted cells were resuspended in 3 to 4 ml of cryopreservation medium by gently pipetting. Appropriate aliquots of the cell suspension were added to new cryopreservation vial (Nunc) and stored in and placed in a -70 ℃ freezer overnight. The vial was then transferred into liquid nitrogen (-196 ℃). Cells were cryopreserved so that a new stock could be revived in case the maintained cells became contaminated.

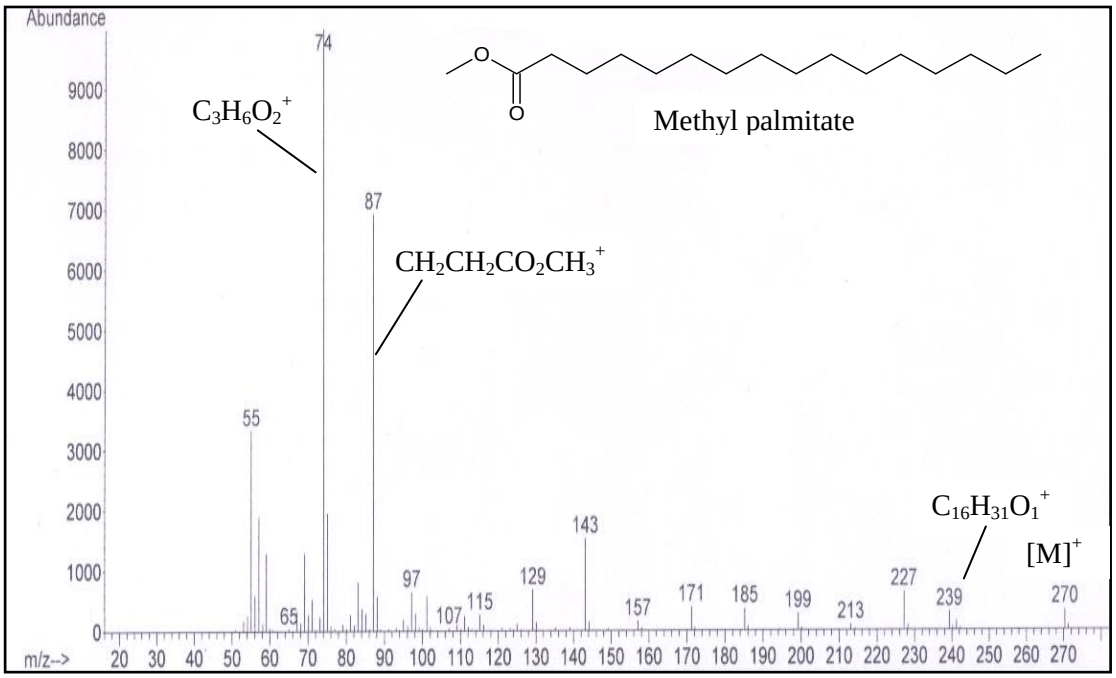
Appendix B

Gas chromatogram of hexane extract of *P. bleo*



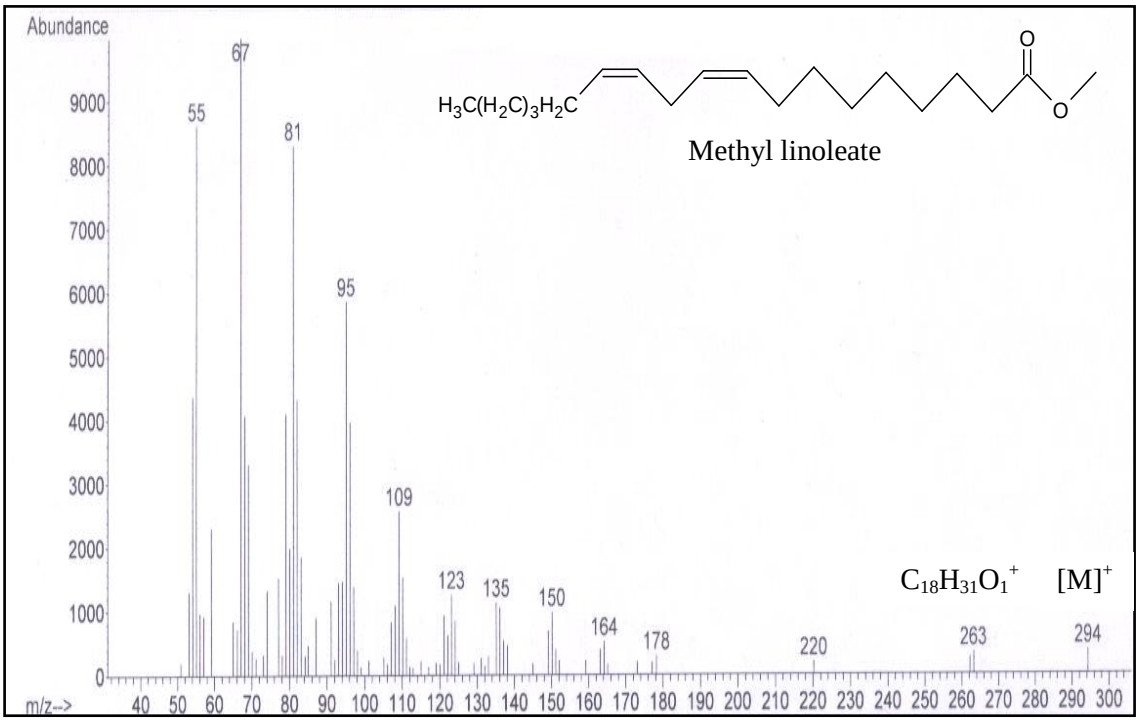
Appendix B1

Mass spectrum of methyl palmitate



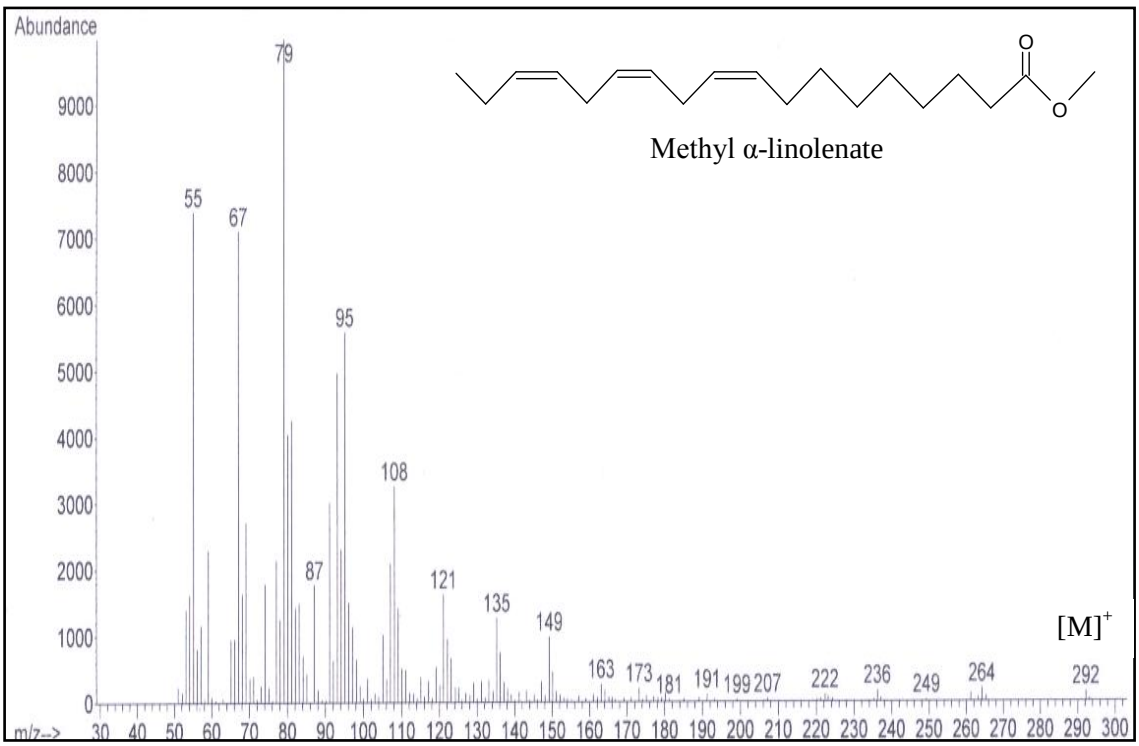
Appendix B2

Mass spectrum of methyl linoleate



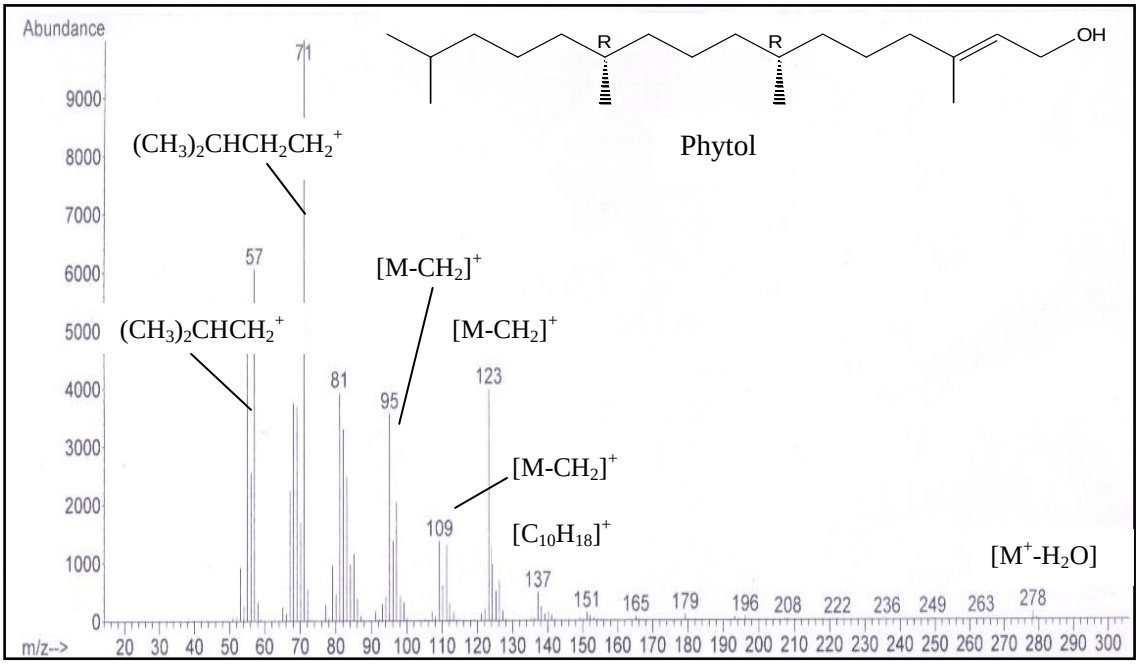
Appendix B3

Mass spectrum of methyl α-linolenate



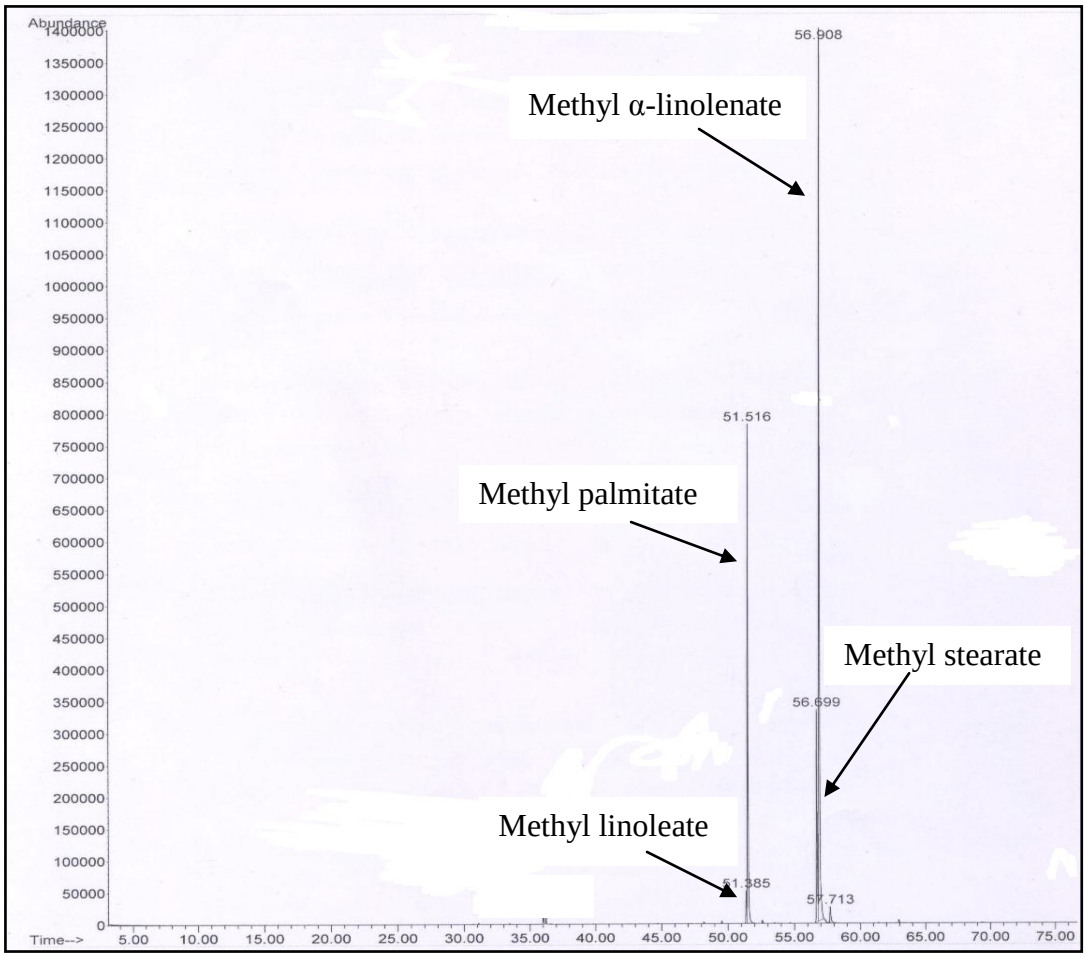
Appendix B4

Mass spectrum of phytol



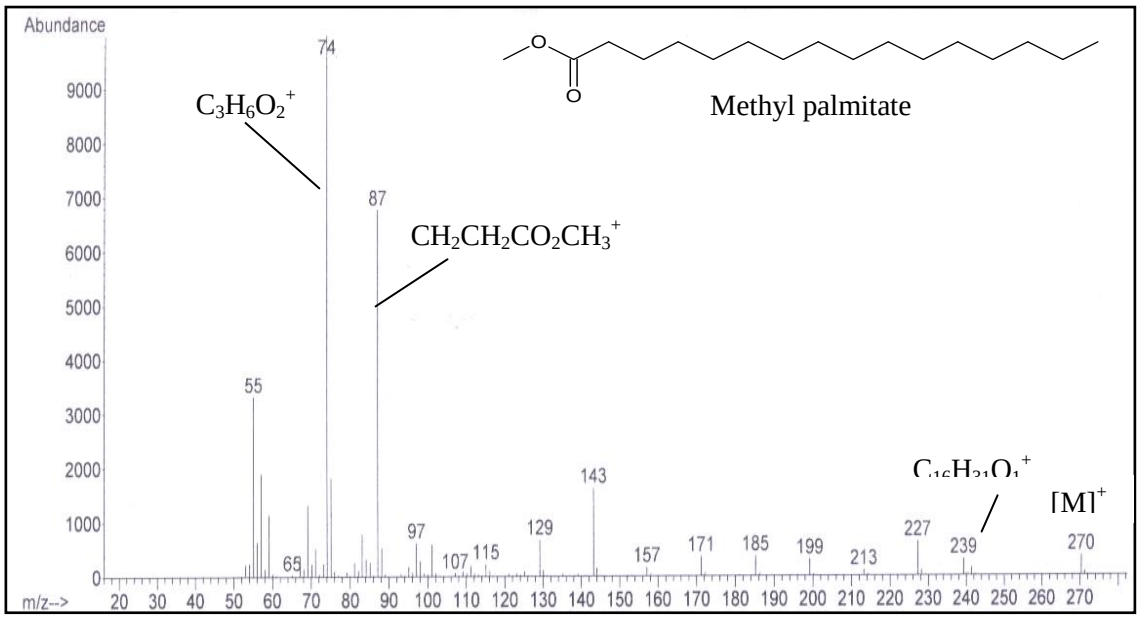
Appendix C

Gas chromatogram of hexane extract of *P. grandifolia*



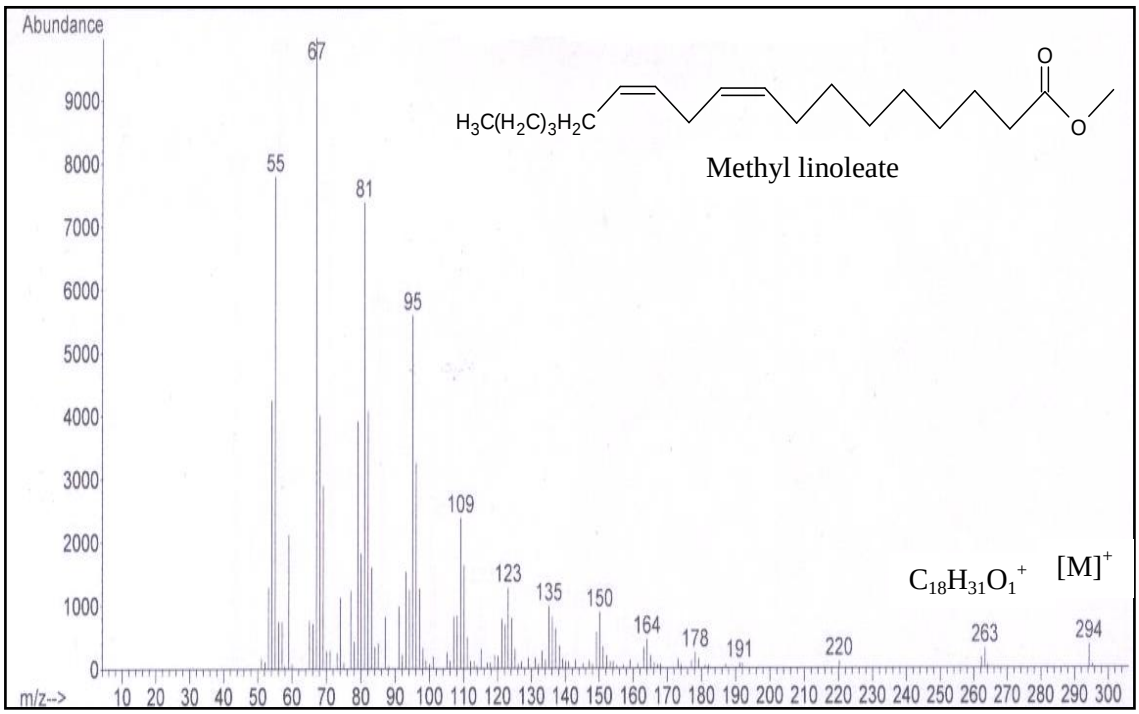
Appendix C1

Mass spectrum of methyl palmitate



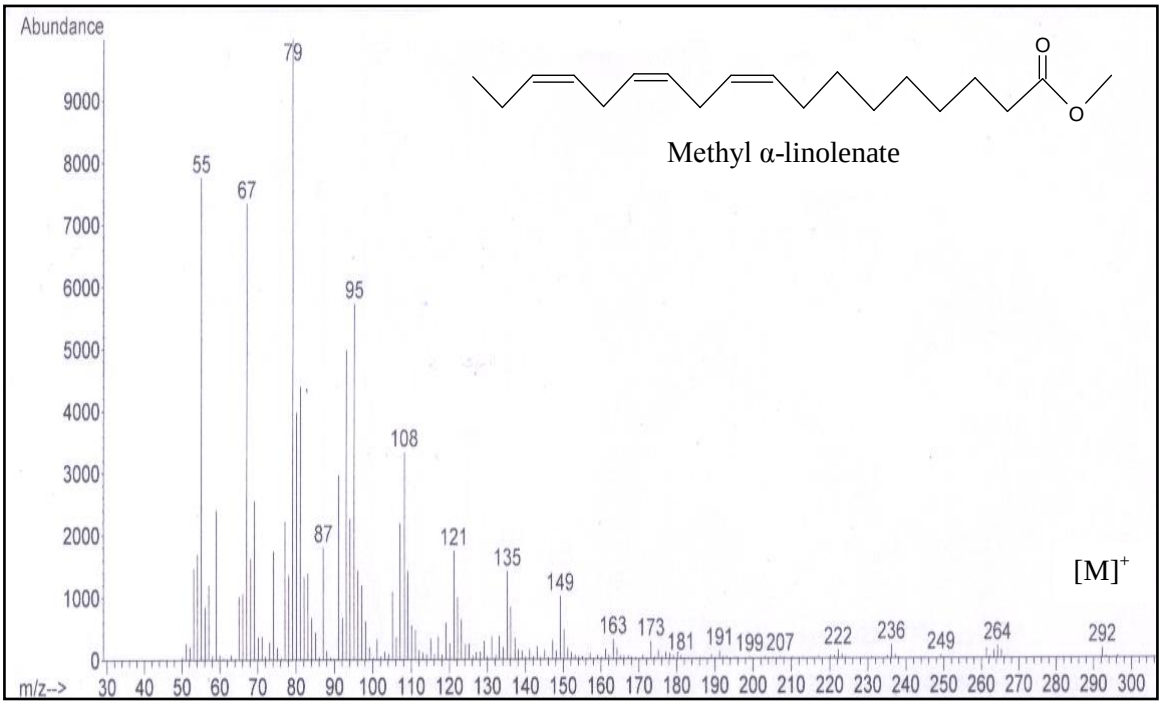
Appendix C2

Mass spectrum of methyl linoleate



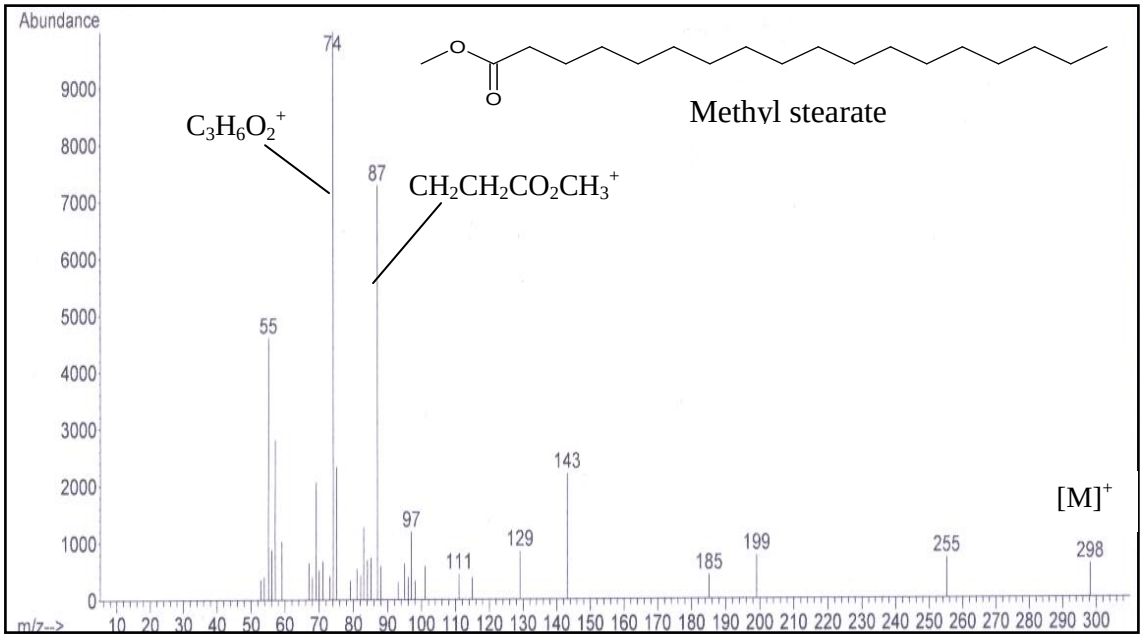
Appendix C3

Mass spectrum of methyl α-linoleate



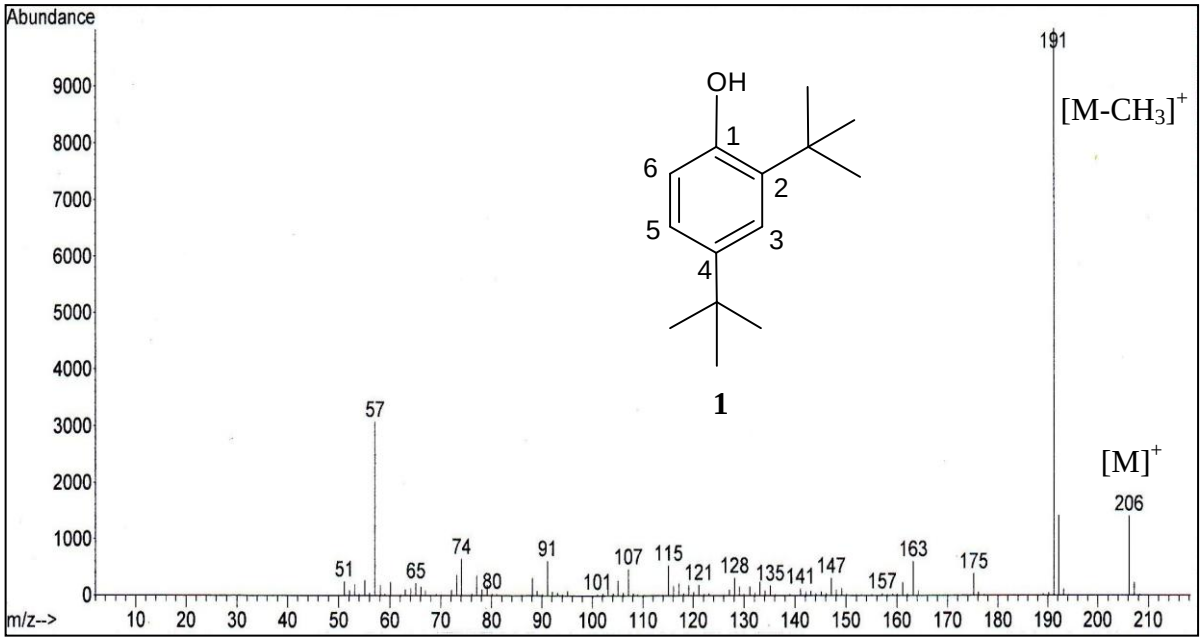
Appendix C4

Mass spectrum of methyl stearate



Appendix D

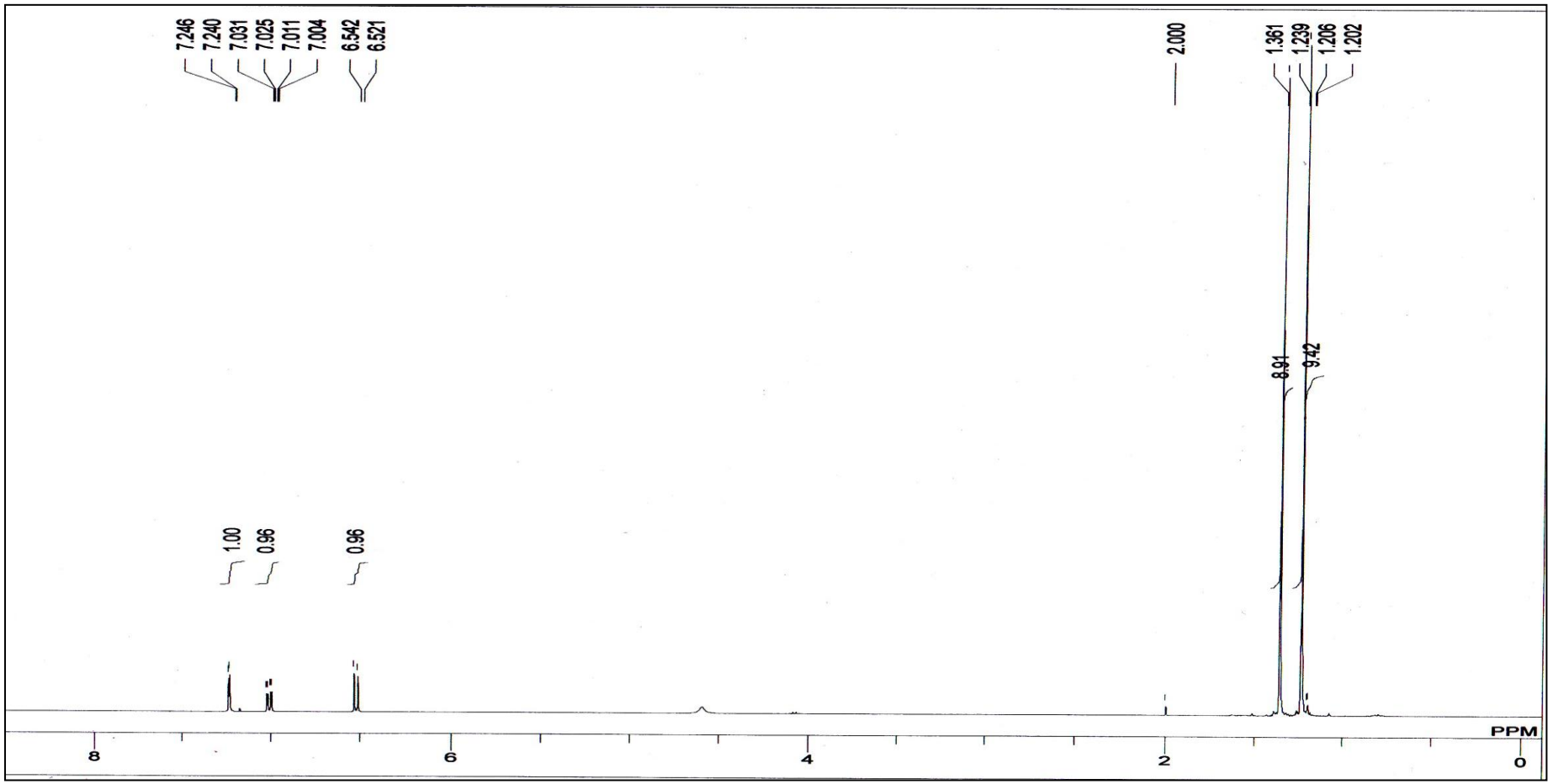
Mass spectrum of 2,4-di-tert-butylphenol (1)



1

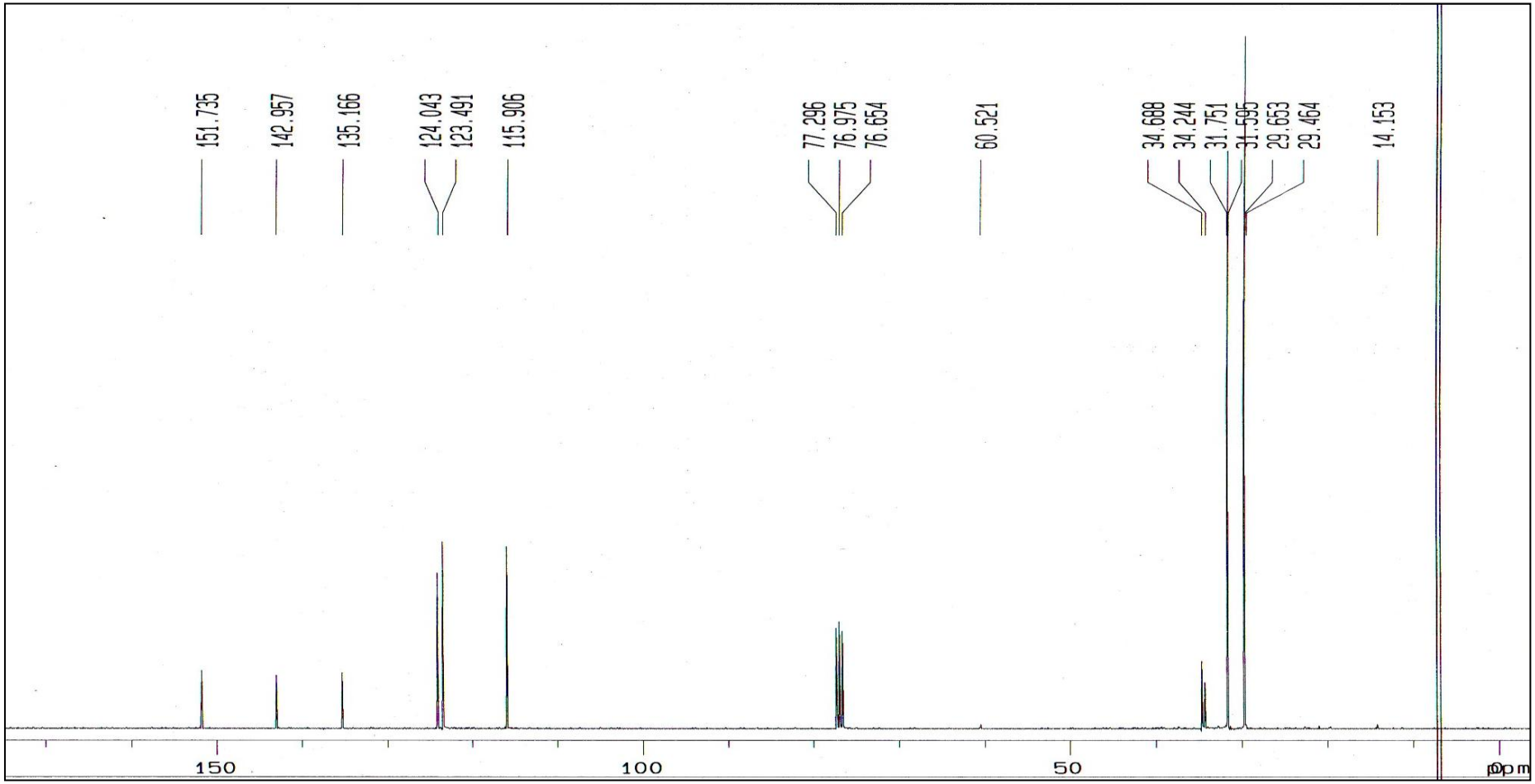
Appendix D1

^{1}H-NMR spectrum of 2,4-di-tert-butylphenol (1)



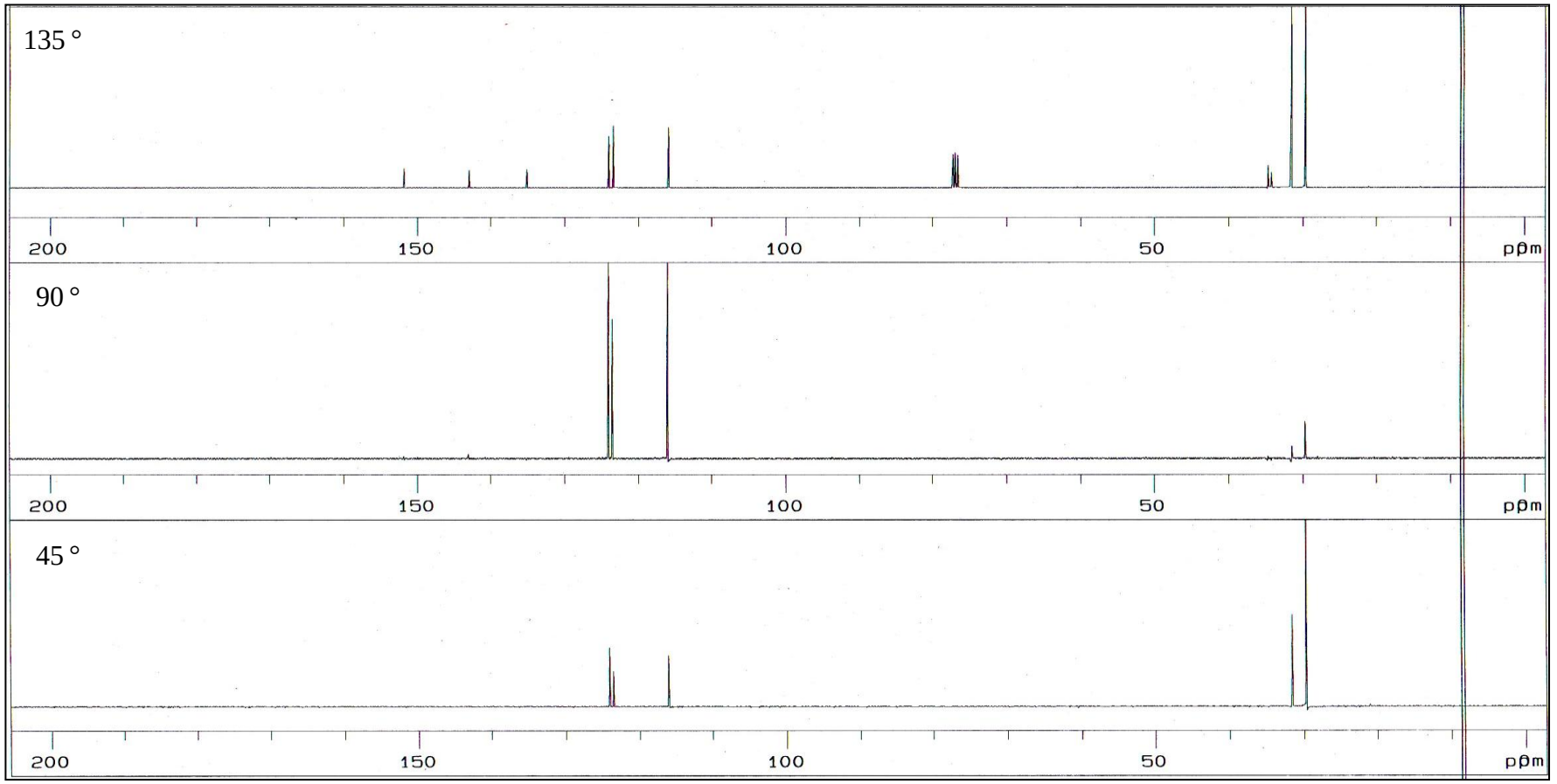
Appendix D2

^{13}C-NMR spectrum of 2,4-di-tert-butylphenol (1)



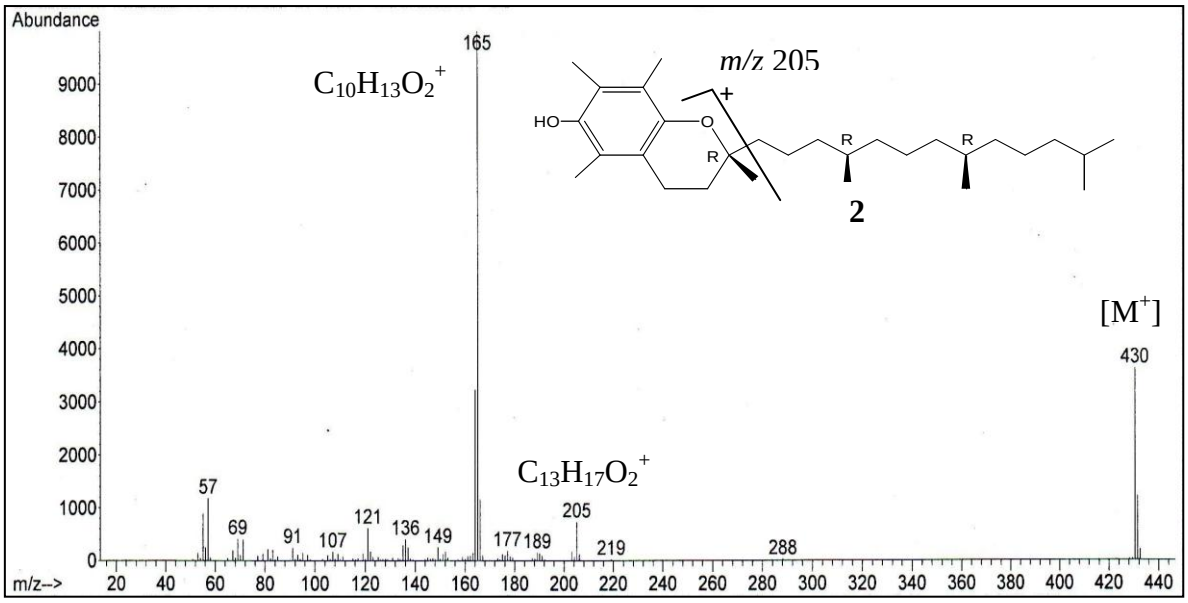
Appendix D3

DEPT spectrum of 2,4-di-tert-butylphenol (1)



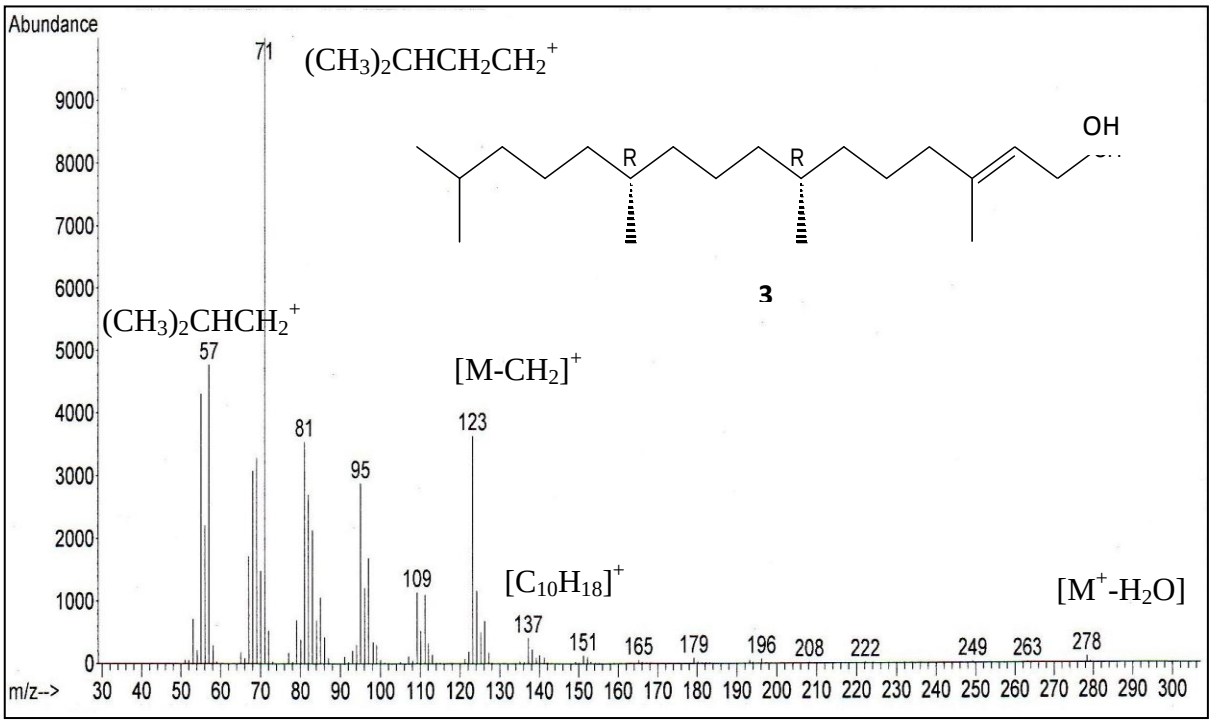
Appendix E

Mass spectrum of α-tocopherol (2)



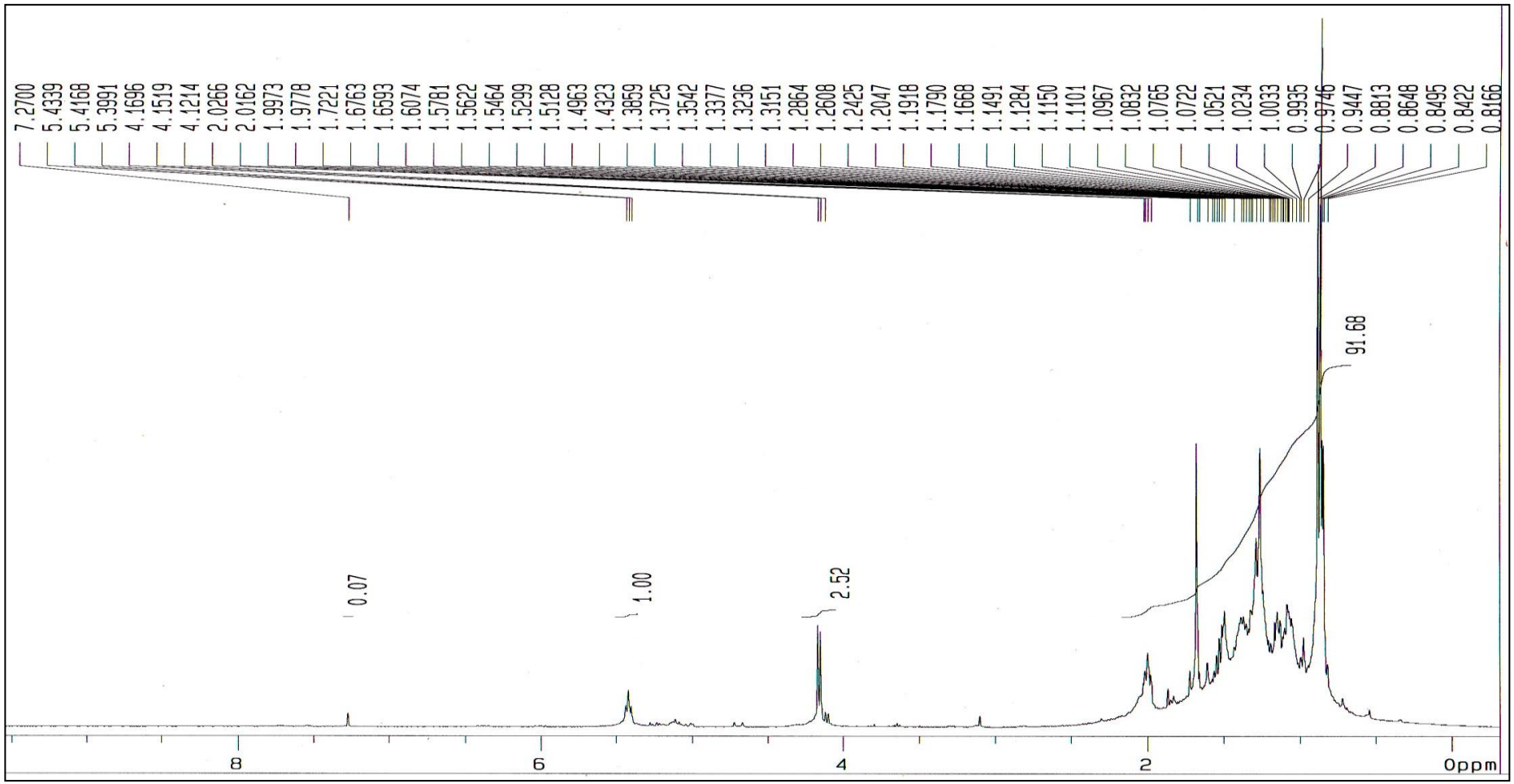
Appendix F

Mass spectrum of phytol (3)



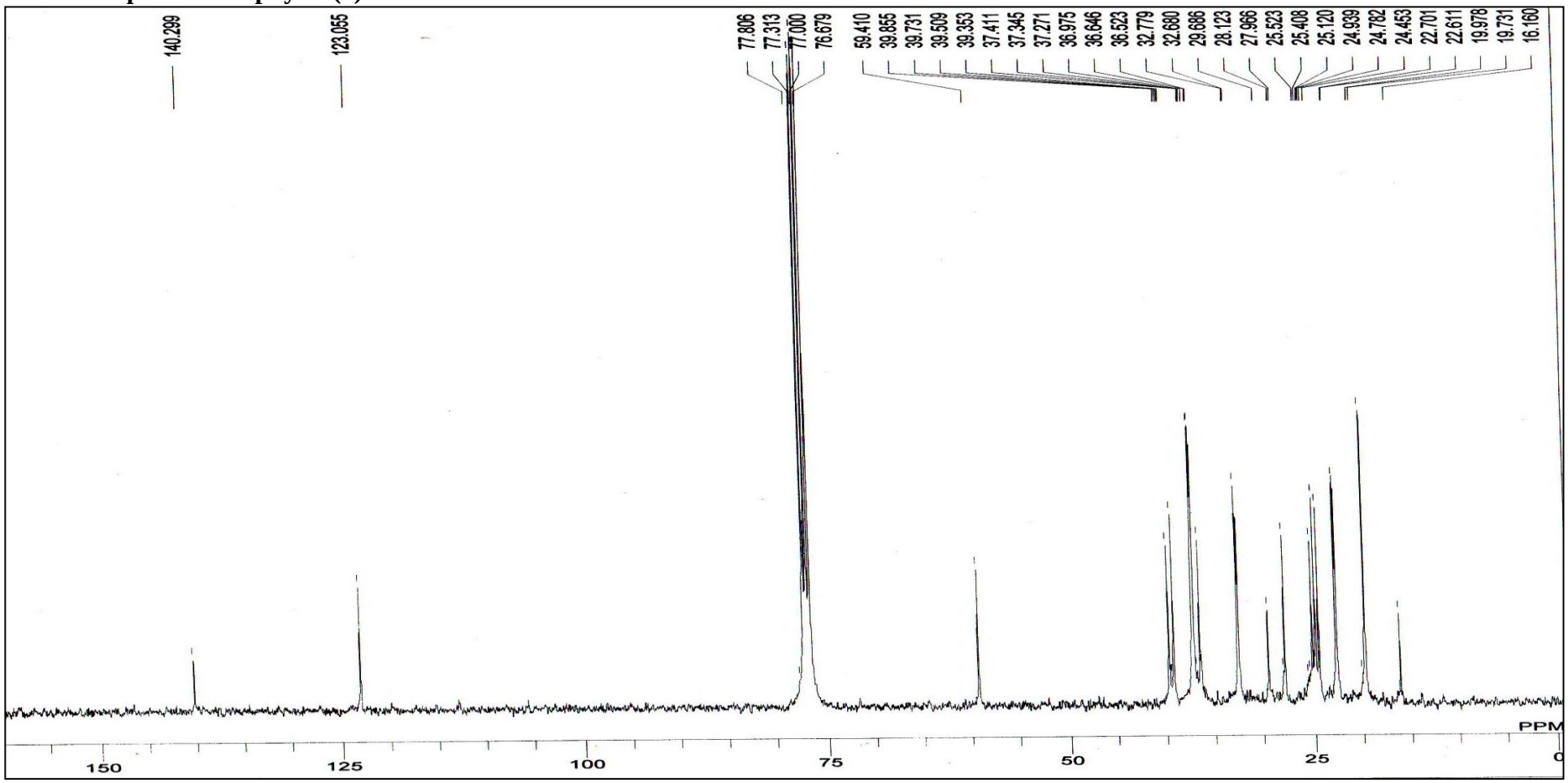
Appendix F1

^{1}H-NMR spectrum of phytol (3)



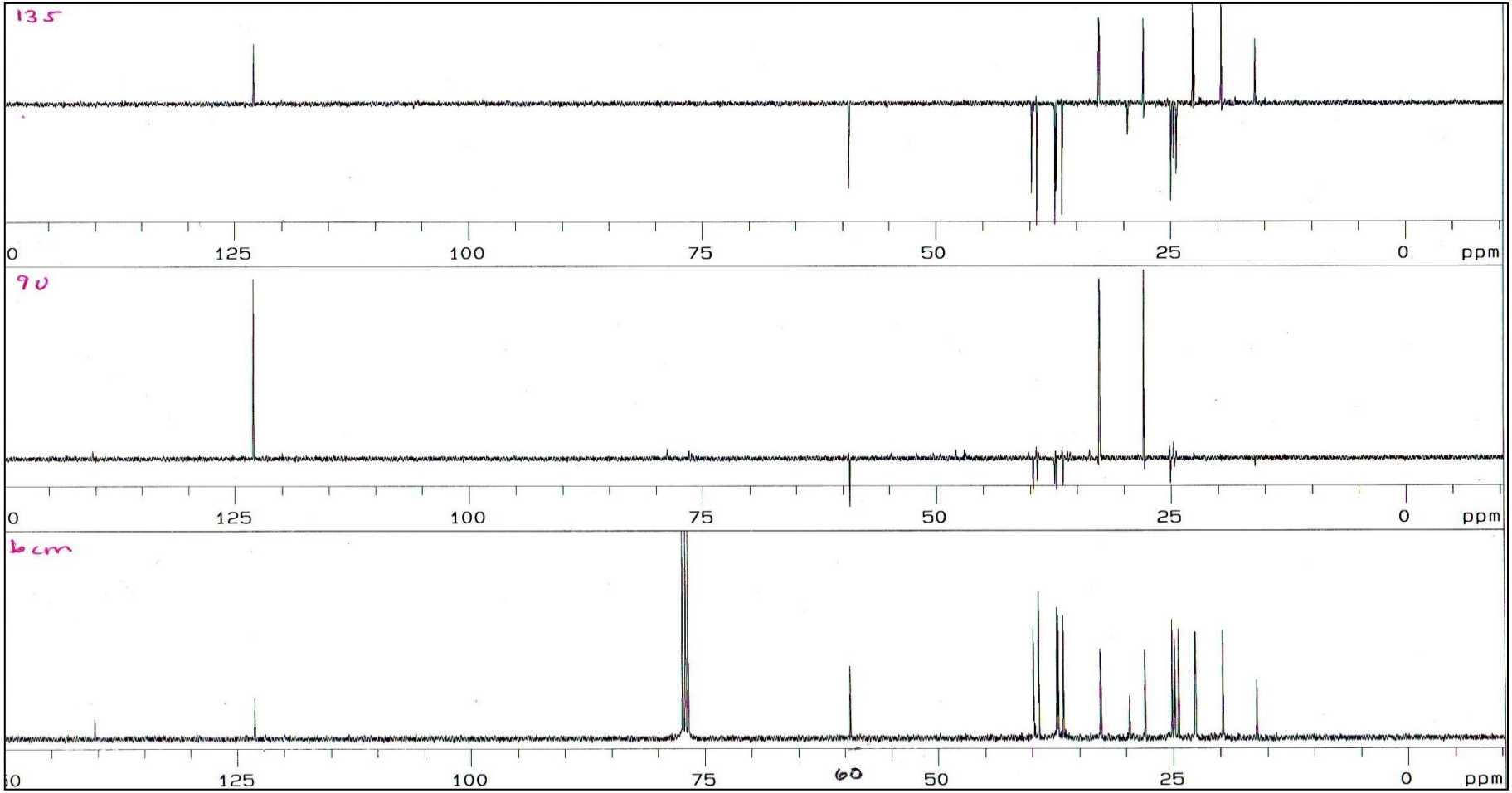
Appendix F2

^{13}C-NMR spectrum of phytol (3)



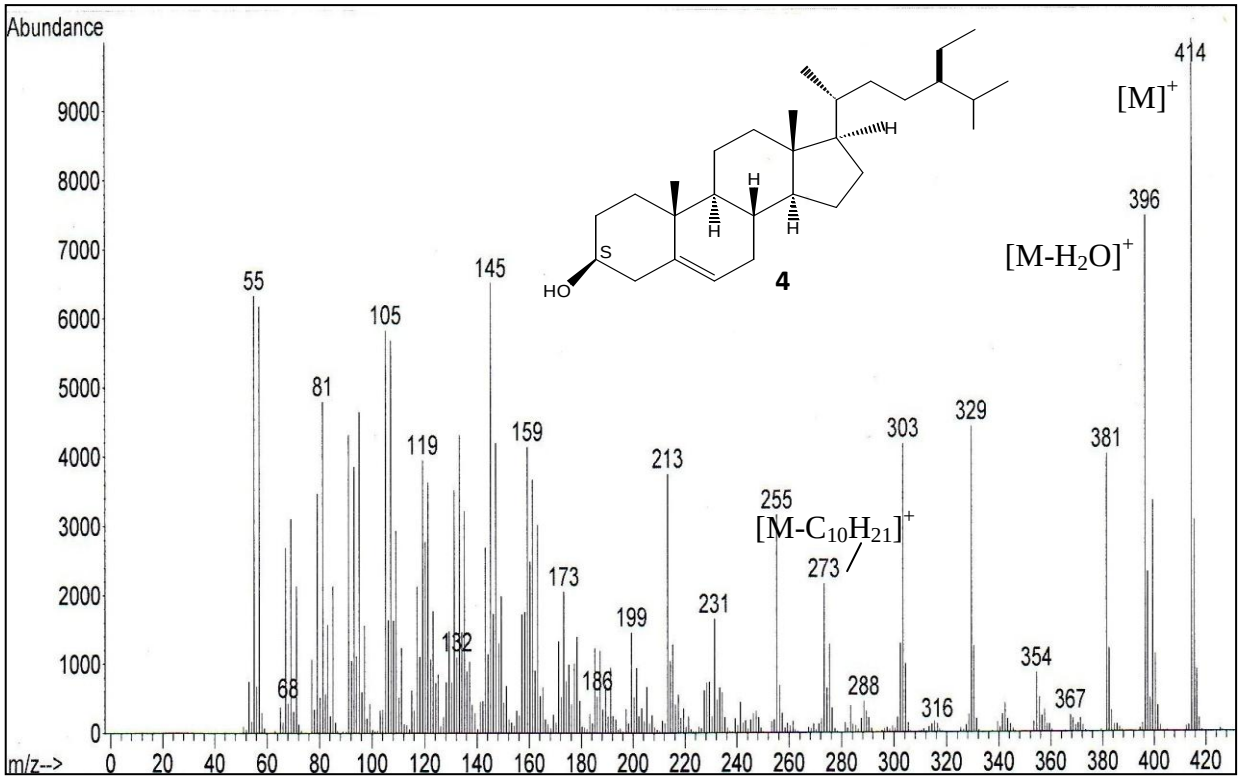
Appendix F3

DEPT spectrum of phytol (3)



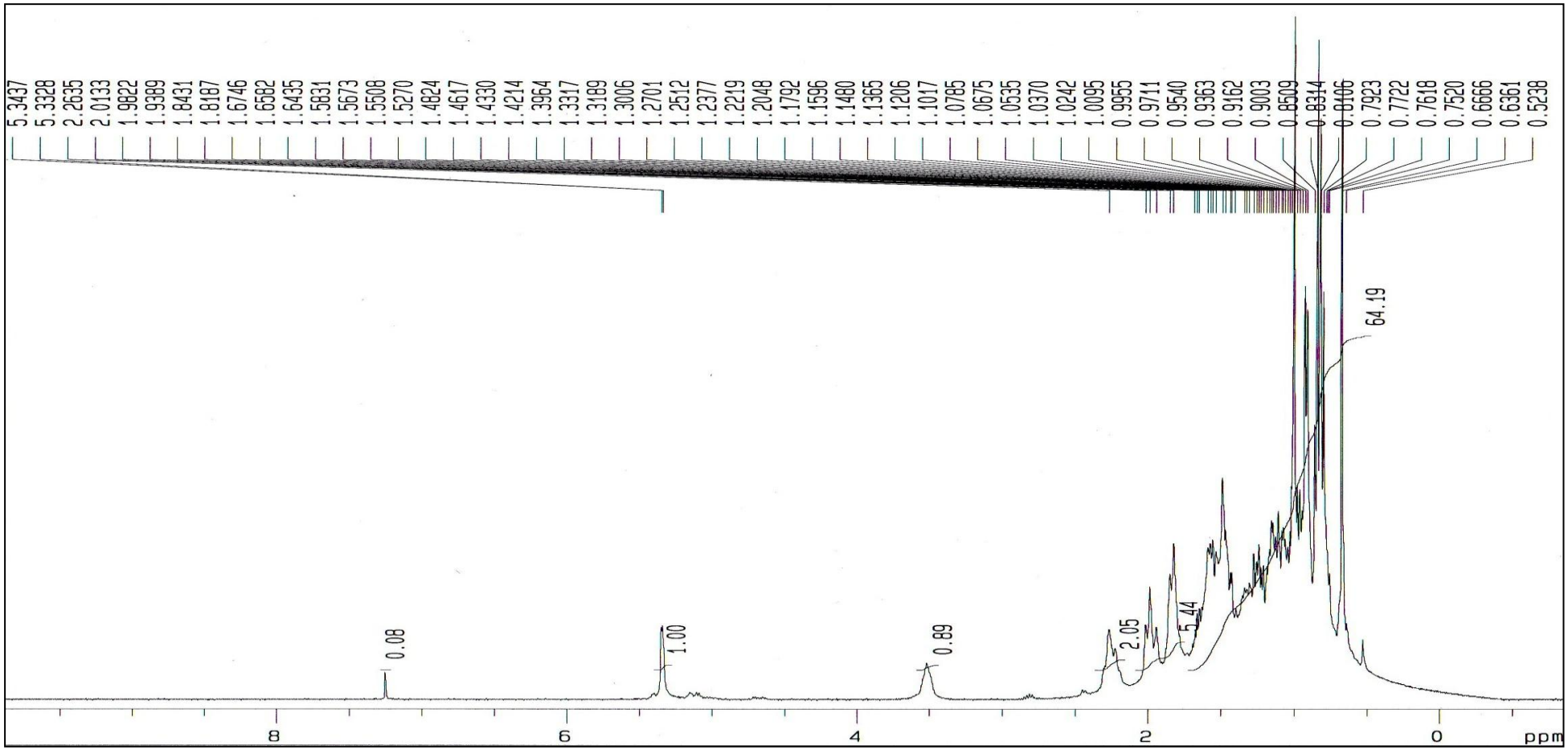
Appendix G

Mass spectrum of ß-sitosterol (4)



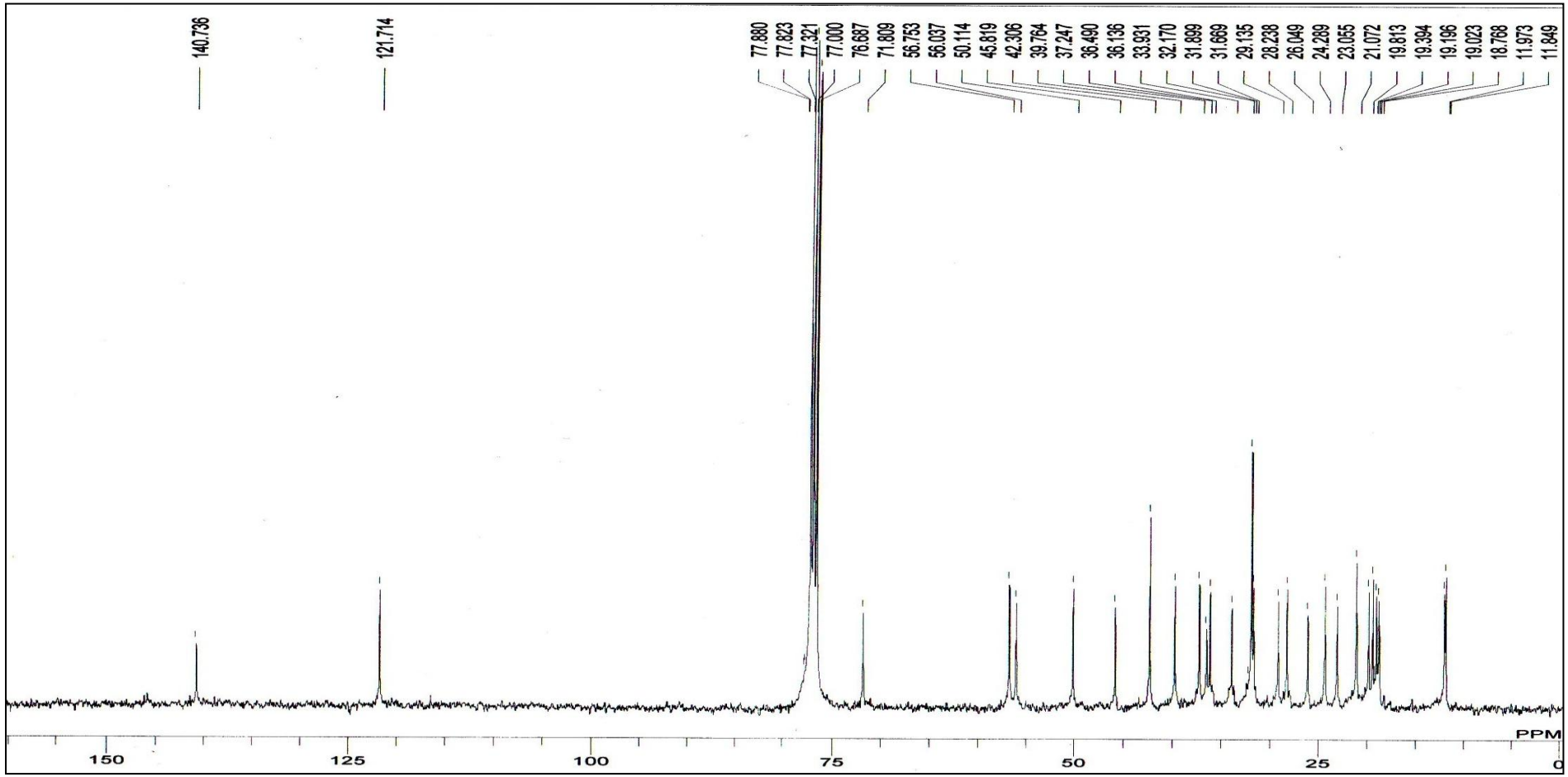
Appendix G1

^{1}H-NMR spectrum of ß-sitosterol (4)



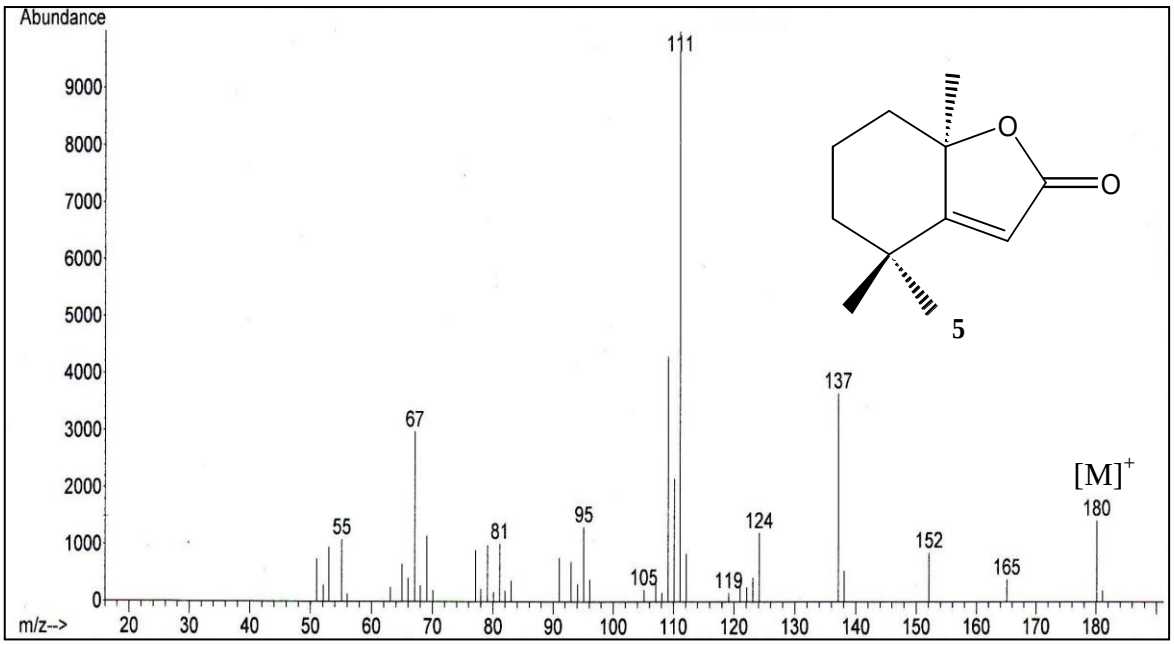
Appendix G2

^{13}C-NMR spectrum o f ß-sitosterol (4)



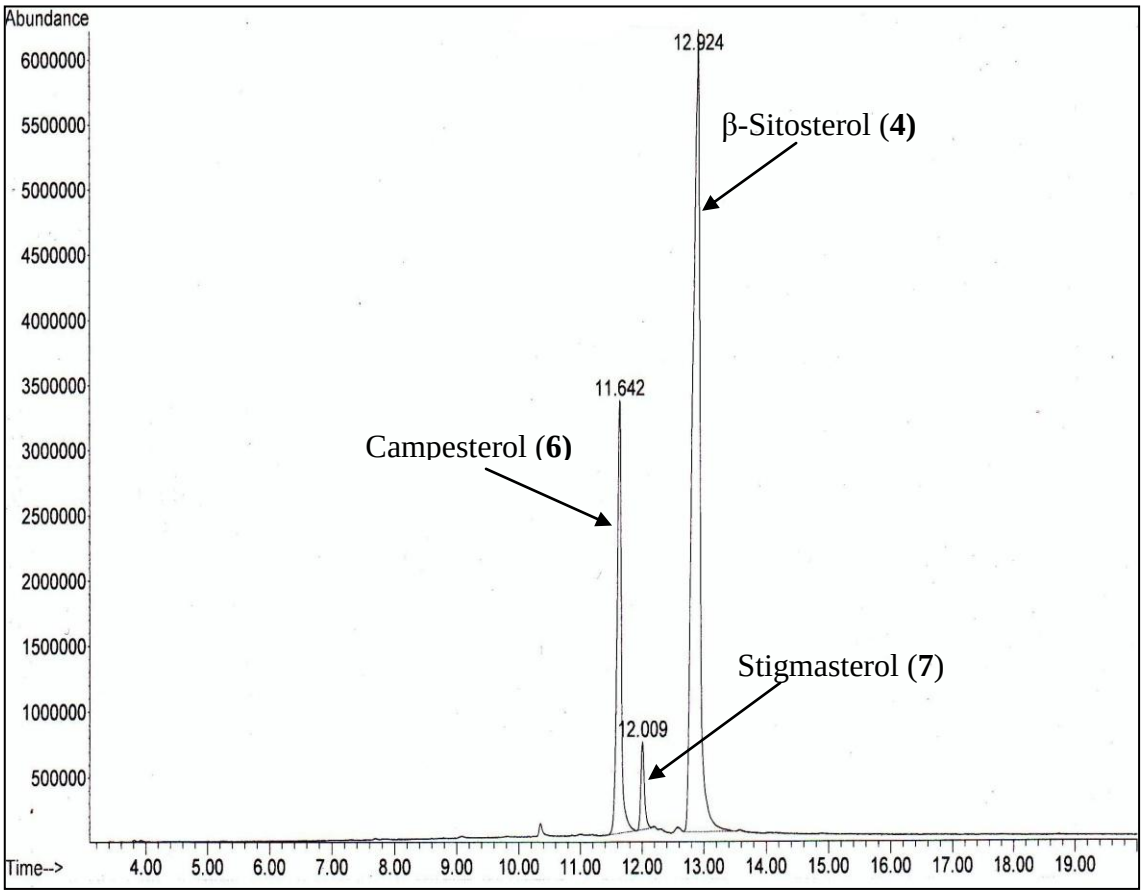
Appendix H

Mass spectrum of dihydroactinidiolide (5)



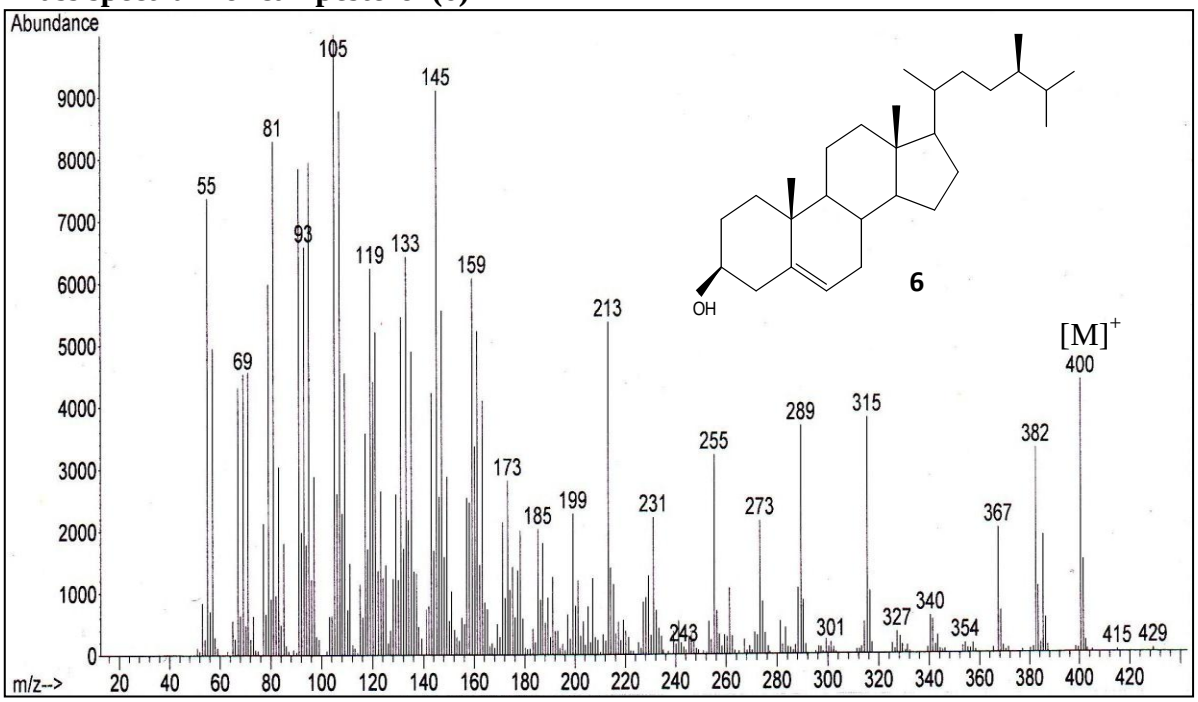
Appendix I

Gas chromatogram of mixture A



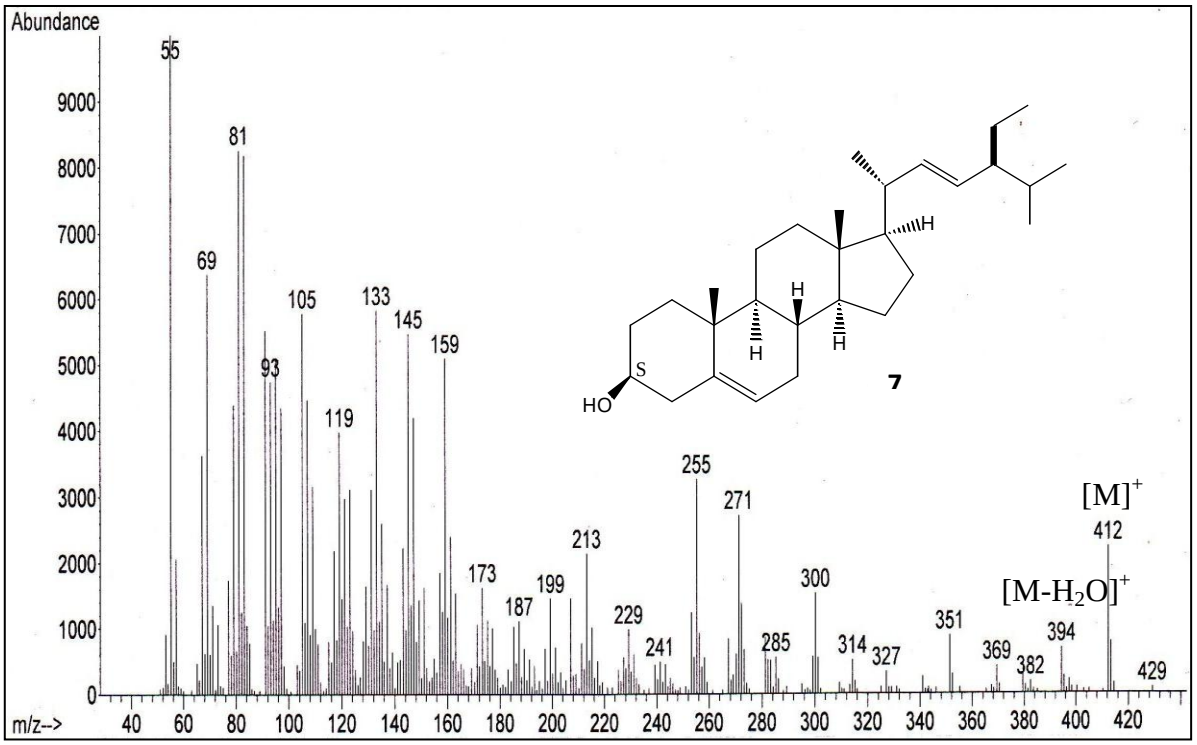
Appendix I1

Mass spectrum of campesterol (6)



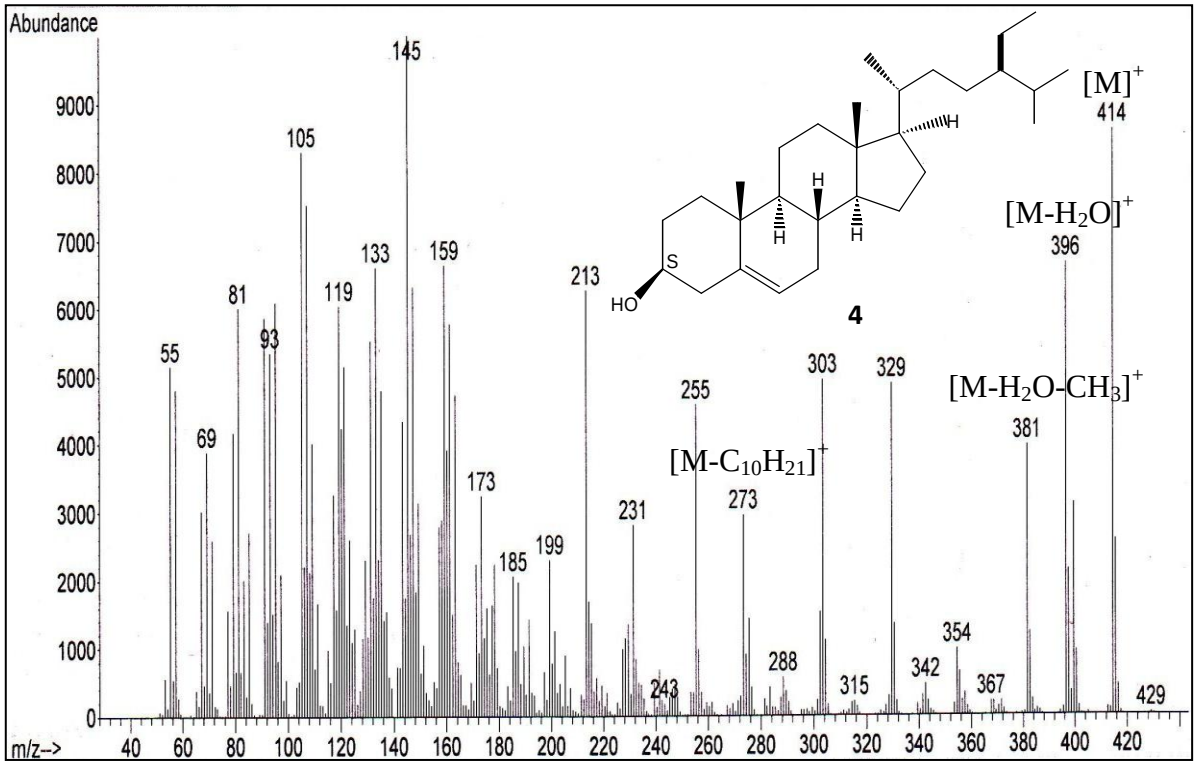
Appendix I2

Mass spectrum of stigmasterol (7)



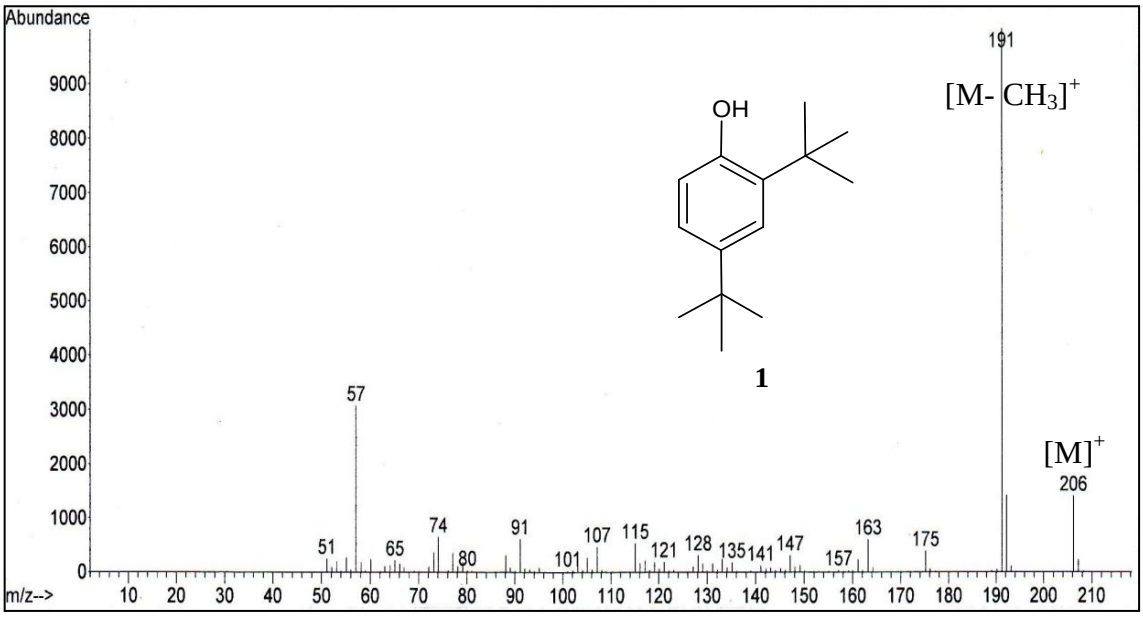
Appendix I3

Mass spectrum of ß-sitosterol (4)



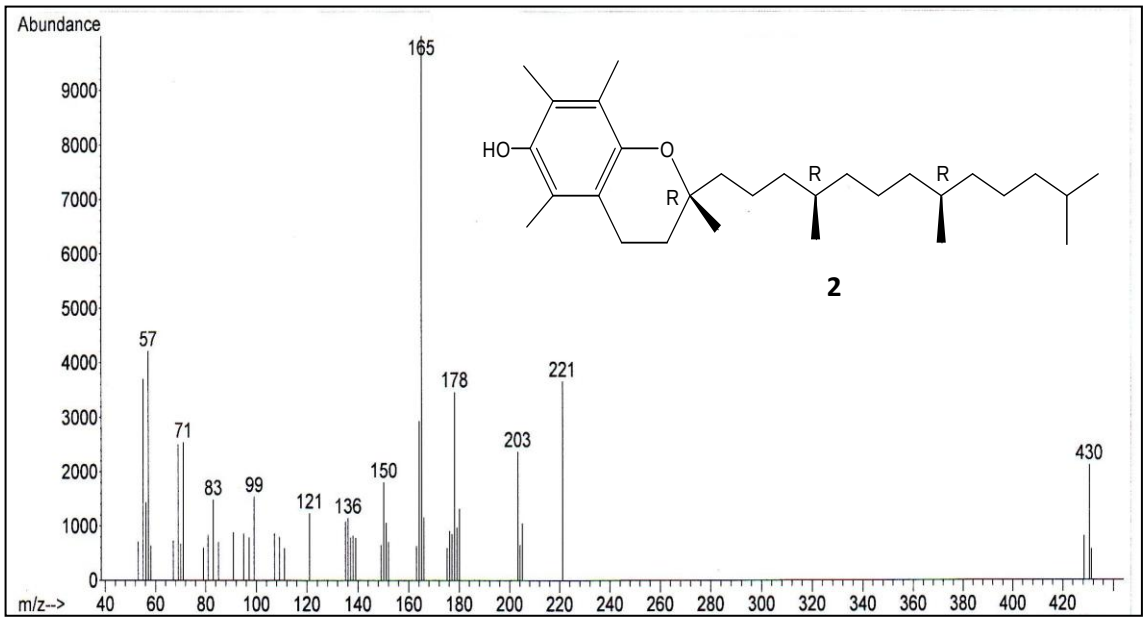
Appendix J

Mass spectrum of 2,4-di-tert-butylphenol (1)



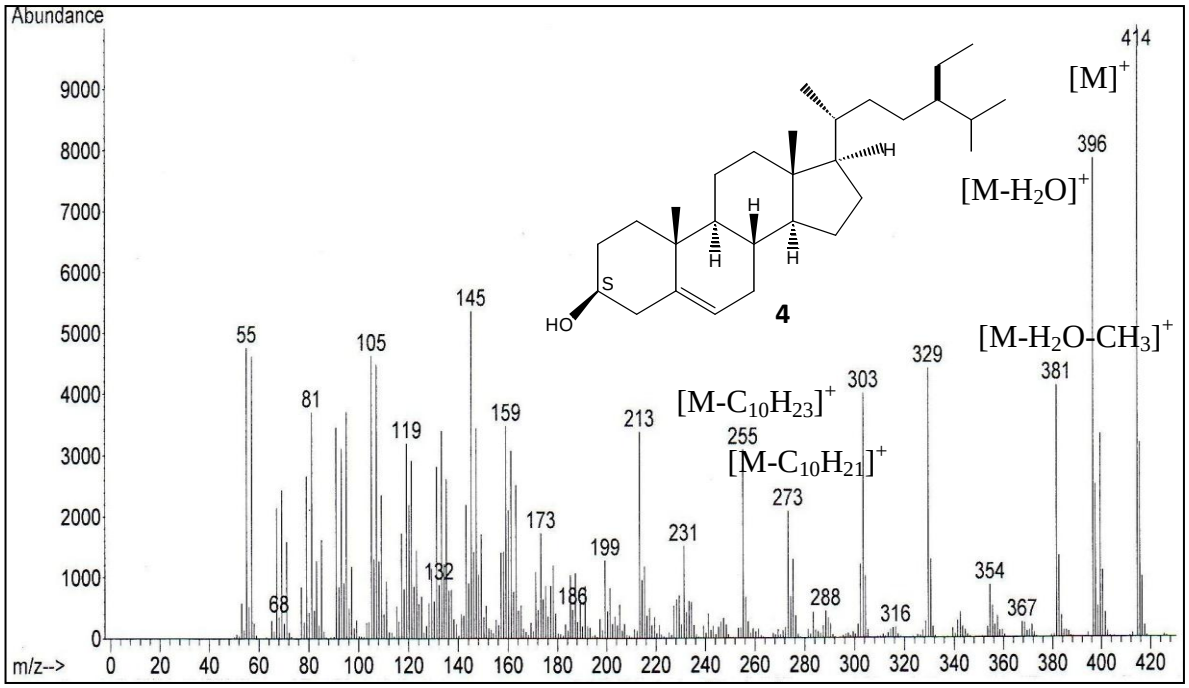
Appendix K

Mass spectrum of α-tocopherol (2)



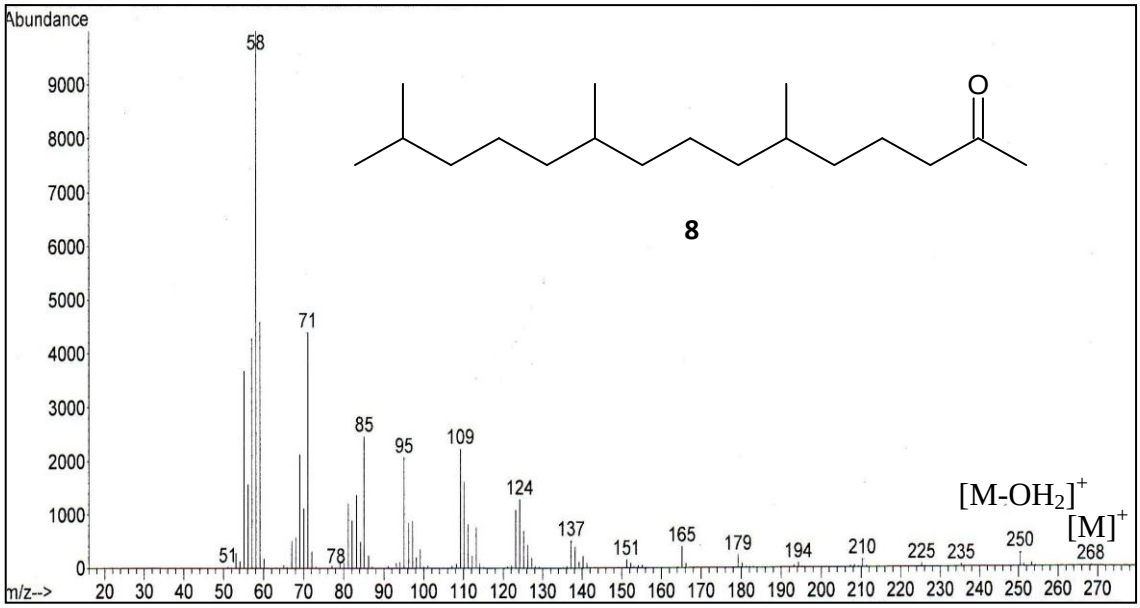
Appendix L

Mass spectrum of ß-sitosterol (4)



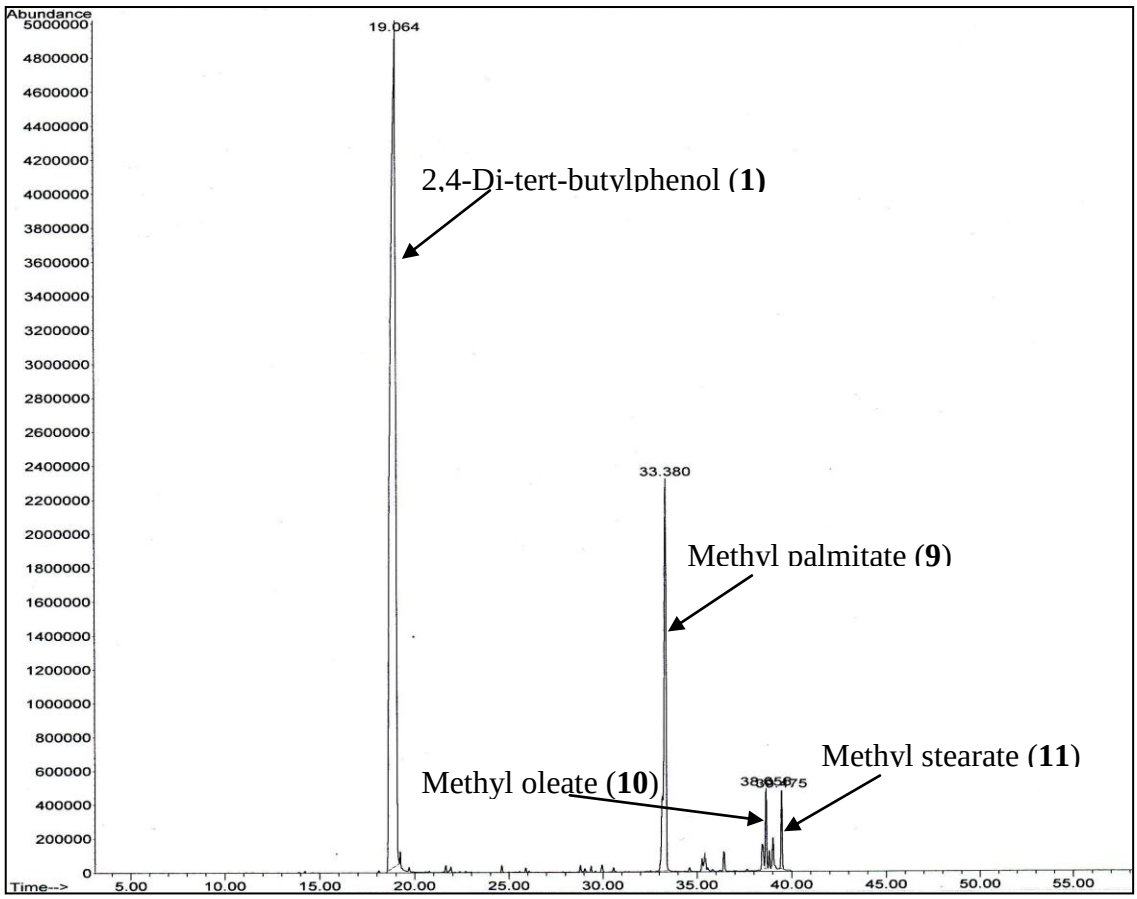
Appendix M

Mass spectrum of phytone (8)



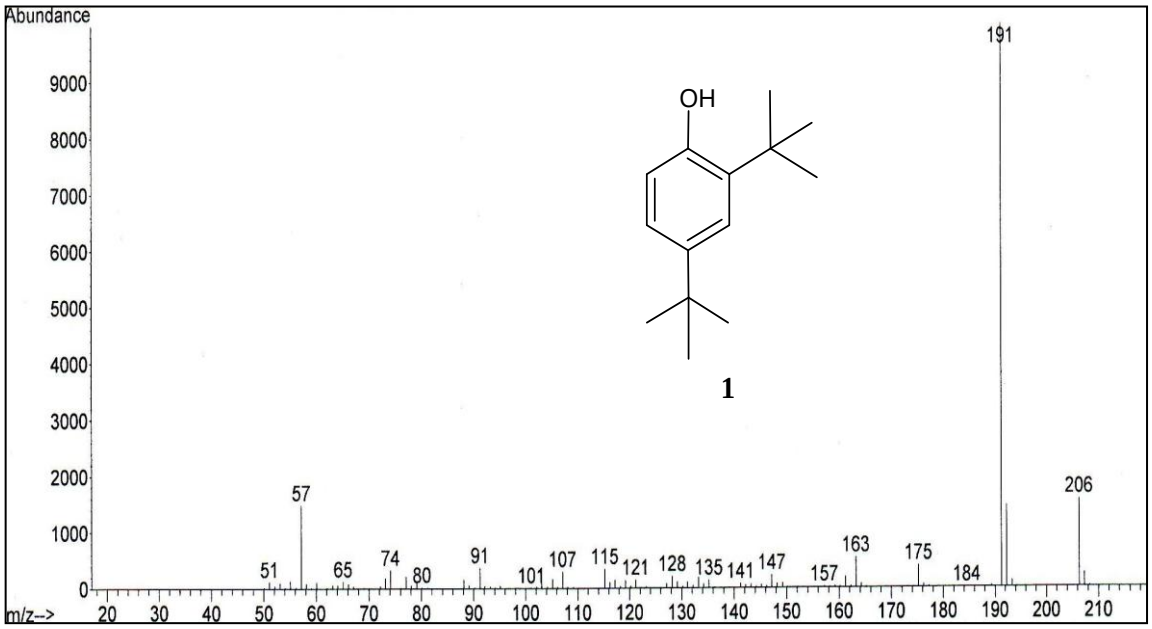
Appendix N

Gas chromatogram of mixture B



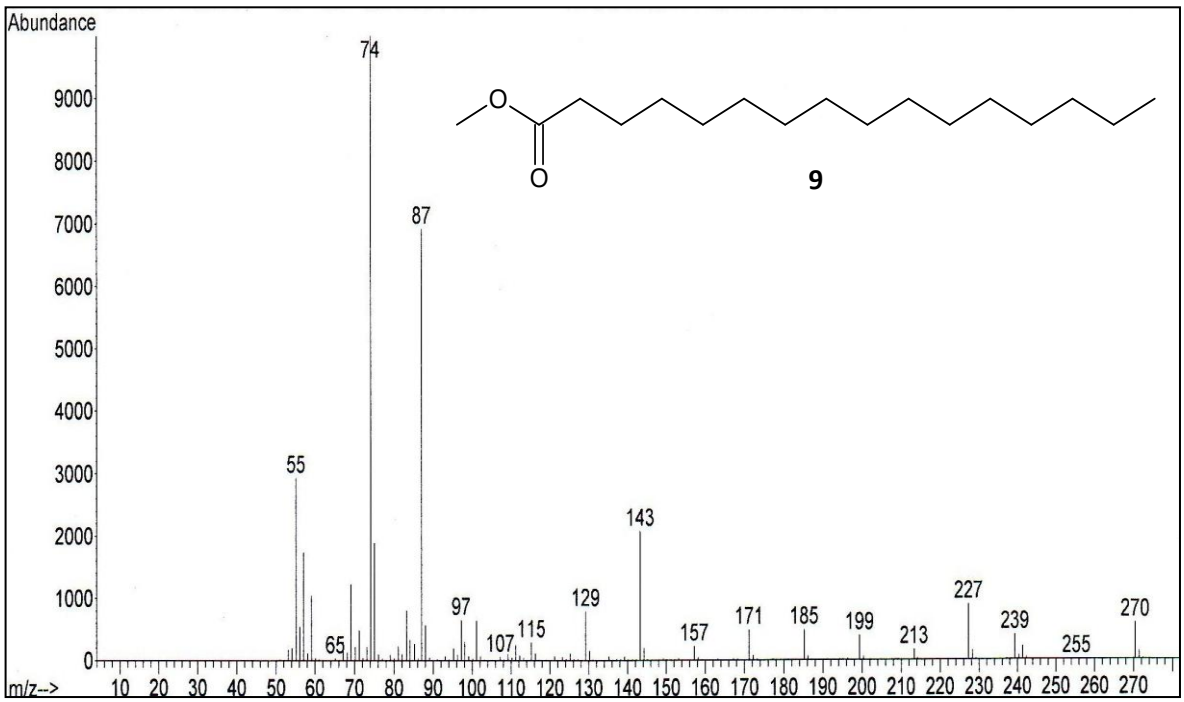
Appendix N1

Mass spectrum of 2,4-di-tert-butylphenol (1)



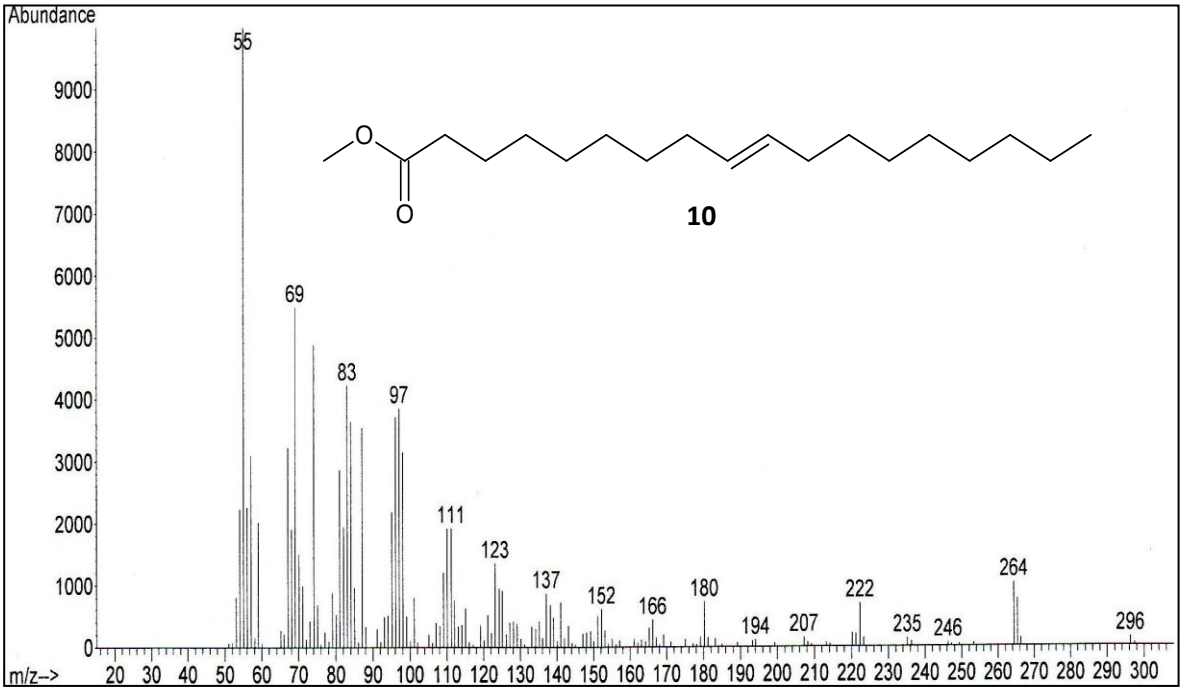
Appendix N2

Mass spectrum of methyl palmitate (9)



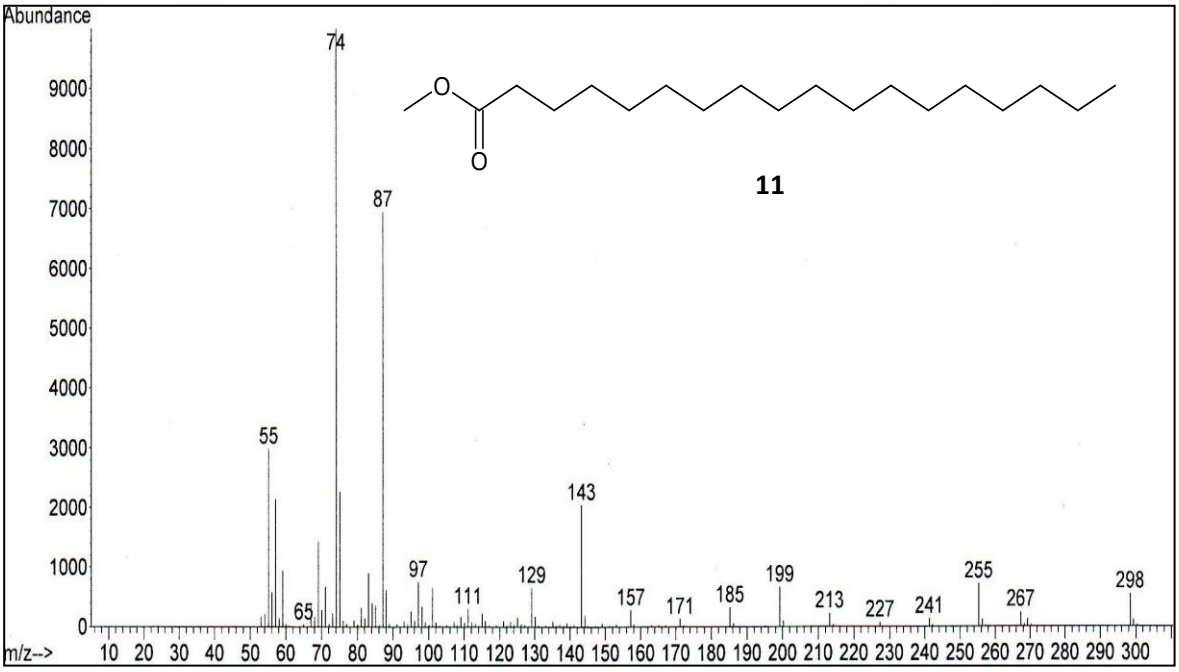
Appendix N3

Mass spectrum of methyl oleate (10)



Appendix N4

Mass spectrum of methyl stearate (11)



Appendix O

List of publications

Some of the data presented in this thesis have been published in international scientific journals and proceedings of conferences as listed below.

a) Publications in international scientific journals

i. Sri Nurestri Abdul Malek, Norhanom Abdul Wahab, Hashim Yaacob, **Sim Kae Shin**, Hong Sok Lai, Lee Guan Serm and Syarifah N.S.A. Rahman. (2008). Cytotoxic activity of *Pereskia bleo* (Cactaceae) against selected human cell lines. *International Journal of Cancer Research*. **4** (1): 20-27.

ii. Sri Nurestri Abdul Malek, **Sim Kae Shin**, Norhanom Abdul Wahab and Hashim Yaacob. (2009). Cytotoxic components of *Pereskia bleo* (Kunth) DC. (Cactaceae) leaves. *Molecules*. **14**: 1713-1724.

iii. Sri Nurestri, A.M., **Sim, K.S.** and Norhanom, A.W. (2009). Phytochemical and cytotoxic investigations of *Pereskia grandifolia* Haw. (Cactaceae) leaves. *Journal of Biological Sciences*. **9** (5): 488-493.

iv. Koshy Philip, Sri Nurestri Abdul Malek, **Sim Kae Shin**, Saravana Kumar, Hong Sok Lai, Lee Guan Serm, Syarifah N.S.A. Rahman and Gowri Kanagasabapathy. (2009). Antimicrobial activity of some medicinal plants from Malaysia. *American Journal of Applied Sciences*. **6** (8): 1613-1617.

v. **Sim, K.S.**, Sri Nurestri, A.M., Sinniah, S.K., Kim, K.H. and Norhanom, A.W. (2010). Acute oral toxicity of *Pereskia bleo* and *Pereskia grandifolia* in mice. *Pharmacognosy Magazine*. **6** (21): 67-70.

vi. **Sim K.S**., Sri Nurestri A.M. and Norhanom A.W. (2010). Phenolic content and antioxidant activity of crude and fractionated extracts of *Pereskia bleo* (Kunth) DC. (Cactaceae). *African Journal of Pharmacy and Pharmacology*. **4** (5): 193-201.

vii. **Sim K.S.,** Sri Nurestri A.M. and Norhanom A.W. (2010). Phenolic content and antioxidant activity of *Pereskia grandifolia* Haw. (Cactaceae) extracts. *Pharmacognosy Magazine*. (Accepted).

b) Publications in proceedings of international conference / seminar / workshop

i. **Shin, Sim K**., Malek, Sri. N.A**.**, Yaacob, Hashim, Wahab, Norhanom A., Serm, Lee G., Lai, Hong S., Rahman, Syarifah N.S.A. and Hadi, A.H.A. (2006). Cytotoxic Activity of *Pereskia corrugata* Against Selected Cell Lines. In: The 12th Asian Symposium on Medicinal Plants, Spices and Other Natural Products (ASOMPS XII), Padang, West Sumatera, Indonesia, 13 - 18 November 2006, Program and Abstracts pg. 108.

ii. Sri Nurestri Abd Malek, **Sim Kae Shin**, Koshy Philip, Hong Sok Lai, Lee Guan Serm, Syarifah Nur Syed Abdul Rahman and Gowri Kanagasabapathy. (2008). Antimicrobial activity of eight medicinal plants from Malaysia. In: 2nd Penang International Conference for Young Chemists, Universiti Sains Malaysia, Penang, Malaysia, 18 – 20 June 2008, Abstract Book pg. 395.

iii. **Sim Kae Shin**, Sri Nurestri Abdul Malek and Norhanom Abd. Wahab. (2008). Cytotoxic activity of *Pereskia grandifolia* against selected cancer cell lines. In: 13th Biological Sciences Graduate Congress, National University of Singapore, Singapore, 15 - 17 December 2008, Program and Abstracts pg. 62.

iv. **Sim Kae Shin**, Sri Nurestri Abdul Malek and Norhanom Abd. Wahab. (2009). Investigation on the antioxidant activity of *Pereskia bleo* and *Pereskia grandifolia*. In: 7th COSTAM/SFRR (Asia/Malaysia) International Workshop, Meritus Pelangi Beach Resort & Spa, Langkawi, Malaysia, 9 – 12 July 2009, Program and Abstracts pg. 206.

c) Publications in proceedings of national conference / seminar / workshop

i. Sri Nurestri Abdul Malek, Norhanom Abdul Wahab, **Sim Kae Shin**, Hong Sok Lai, Syarifah Nur Syed Abdul Rahman, Lee Guan Serm and Gowri Kanagasabapathy. (2009). Bioactivity and chemical investigations of *Pereskia bleo*. In: Ekspo Penyelidikan Rekacipta Inovasi, University of Malaya, Malaysia, 13-15 January 2009, Direktori pg. 26.